SERDP CU-1129 BIOLOGICAL ASSESSMENT FOR CHARACTERIZING CONTAMINANT RISK AT THE GENETIC-, INDIVIDUAL-, AND POPULATION-LEVEL

Final Technical Report

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1. BACKGROUND

Currently there is a lack of defensible methods that can be used to measure and assess ecosystem responses to DOD relevant contaminants. Cleanup goals for contaminated DOD sites are largely based on contaminant levels derived by solvent extraction, instrumental analyses, and assumptions about possible exposure pathways and contaminant toxicity. Large uncertainties surround current estimates of environmental risk because of the current dependence on chemically based approaches. These uncertainties result from assumptions regarding contaminant bioavailability, the toxicity of military-relevant contaminants, the toxicity of complex mixtures, and extrapolating to higher order effects (e.g., population-level impacts). The large assumptions and extrapolations required by current approaches necessitate the use of large safety/uncertainty factors which lead to very conservative cleanup goals that are very expensive to obtain with current cleanup technologies.

2. OBJECTIVE

The objective of this project is to develop a suite of technically defensible assays that can be effectively used in regulatory programs to quantify the ecological risk of contaminated sediments at the molecular-, individual-, and population-level. Effects-based assays will be developed which effectively account for contaminant bioavailability while providing an integrated measure of the toxicity of complex mixtures. Dose-response information will be generated for military-relevant contaminants (e.g., TNT, RDX, HMX) by measuring molecular and whole organism responses. Population-level impacts caused by contaminant exposure will be projected using population models developed during this project. A screening approach will be developed to provide cost-effective information about the potential for impacts at the population-level. In developing this screening approach, we will quantify the biological and ecological significance of toxicant effects on gene expression by way of comparison to whole-organism toxicity and modeled population-level impacts.

3. TECHNICAL APPROACH

During this project we evaluated linkages among responses in gene expression, whole-organism toxicity, and population-level impacts using four organisms currently being used by the USEPA and USACE to develop chronic, sublethal sediment bioassays for national regulatory programs. Two of the species occur in marine habitats (*Neanthes arenaceodentata* and *Leptocheirus plumulosus*) and two of the species are found in freshwater habitats (*Hyalella azteca* and *Chironomus tentans*). By simultaneously measuring biological responses at three distinct levels of biological organization (i.e., genes, whole organisms, populations) we will have the ability to effectively test the reliability of estimating potential risk at higher levels of organization (e.g., populations) using information that can be quickly and inexpensively collected at lower levels of organization (i.e., the level of genes).

Genes responding to explosives exposure were identified using a combination of conserved polymerase chain reaction (PCR) primers and differential display of exposed vs. unexposed organisms. Rapid quantitative real time PCR assays were developed to determine the dose-response relationship between gene expression and explosive exposure in *Leptocheirus plumulosus*. Whole-organism chronic toxicity data for sediments spiked singly with TNT (and two of its degradation products), RDX and HMX were collected. Spiked-sediment studies addressed the toxicological interaction of nitroaromatic compounds. Aqueous toxicity experiments addressed the toxicological interaction of a mixture containing nitroaromatics, a heavy metal and a polycyclic aromatic hydrocarbon. Field-contaminated sediments were used in sediment and porewater toxicity experiments to investigate the use of laboratory-generated single compound data to predict the toxicity of field-collected sediment.

4. SUMMARY

Nitroaromatic (e.g., TNT, TNB) and cyclonitramine (e.g., RDX and HMX) explosive compounds were extensively released to the environment during manufacturing, handling and disposal operations at military sites in the U.S., and throughout the world, resulting in high levels of contamination in surface water, ground water, soils, and sediments. The presence of explosive compounds in the aquatic environment has been related to detrimental impacts on biological communities. Therefore, high environmental concentrations of explosive compounds are potentially damaging to natural ecosystems and represent a significant remediation challenge. We investigated the toxicity of major explosive compounds (i.e., TNT, RDX and HMX) as well as the main breakdown products of TNT, in aqueous and sediment exposures. The explosives RDX and HMX did not decrease invertebrate survival in water or sediment exposures even near water solubility limits or exceedingly high sediment concentrations (>1,000 mg/kg). The aqueous and sediment toxicity of TNT and the TNT breakdown products 2-aminodinitrotolune (2ADNT), 2,4-diaminonitrotoluene (2,4DANT) and trinitrobenzene (TNB) were compared using freshwater invertebrates. TNB was similarly toxic to TNT. Nitro-reduction substantially decreases the toxicity of TNT, as the mono-aminated compound 2ADNT was half as toxic as TNT. Further amination appears to decrease toxicity even more dramatically, as the di-aminated breakdown product 2,4DANT was substantially less toxic than TNT. Such dramatic differences in toxicity among compounds were not observed in exposures to spiked sediments using the midge *Chironomus tentans* or the amphipod *Hyalella azteca*. Results from this investigation indicate that TNT and its daughter compounds interact additively when in a mixture. Therefore, the toxicity of a mixture to a given receptor can be reasonably predicted using single-compound toxicity derived for that receptor. We also investigated the interaction of an explosive compound (TNB) in a mixture with chemicals belonging to different classes of contaminants, more specifically the PAH phenanthrene and the heavy metal lead. Mixture interaction experiments using *C. tentans* suggest that those compounds interact non-additively to promote mortality. Although a less than additive (i.e., antagonistic) toxicological interaction was apparent, further investigation of the mixture interaction among these compound is warranted before definitive conclusions about their mode of interaction are made.

Although aqueous toxicity of TNT has been previously studied for several species of aquatic organisms, the bioaccumulation of TNT in aquatic species is poorly known. We investigated the bioaccumulation of radiolabeled TNT in two benthic invertebrates. Bioconcentration factors derived using ^{14}C-activity as a surrogate for TNT bioaccumulation (~ 100-200 g/ml) were substantially higher than values derived using HPLC-measured water and tissue sum molar concentrations of TNT and its daughter compounds (5.3 – 20.5) in this study. The toxicological significance of the fraction of the body burden that does not correspond to TNT or its major degradation products remains unknown.

We investigated the relationship between compound bioaccumulation and ecologically relevant toxicity endpoints, such as survival, with the objective of deriving critical body residues (CBR) for benthic invertebrates. Critical body residues were determined for *C. tentans* in aqueous exposures to TNT, 2ADNT and 2,4DANT. CBRs, expressed as the sum molar concentrations of chemically detected nitroaromatics, were relatively constant for all compounds, ranging from 0.02 to 0.12 µmol/g. Therefore, it is likely that TNT and its major breakdown products act by similar modes of action in aquatic invertebrates, further supporting the assumption of additivity for the interaction of those compounds.

To better understand effects at the cellular level, genetic assays were developed for assessing effects of contaminants on benthic invertebrates. Using a common sediment bioassay organism, *L. plumulosus,* we isolated genes useful in assessing exposure to bioavailable chemicals in contaminated sediments and waters. Prior to this work virtually no genetic information existed for *L. plumulosus*. We have isolated and identified genes involved in several important biological pathways. These genes include: 26S protease subunit (a component of the principle pathway for degradation of cellular proteins), a QM protein homologue (a novel gene that was first isolated as a putative tumor suppressor gene from a human Wilms' tumor cell line and may have an important biological role associated with development), Mn-superoxide dismutase (controlling oxidative stress and oxygen toxicity by converting the superoxide radical to less dangerous forms), and several families of mobile genetic elements were described for the first time in this work. Mobile genetic elements can cause mutations and chromosomal rearrangements.

Using several rapid gene expression assays, we have correlated expression to sublethal bioaccumulation of TNT and lead. Assays were also found to correlate with CBRs of lead and phenanthrene. By assaying the effects of chemicals on the specific genes, mechanistic inferences can be made about toxic modes of action. TNT exposures induced expression of several genes and mobilized several mobile genetic elements. Low levels of TNT caused a general increase in gene expression, with expression decreasing prior to lethal effects being observed. Several mobile genetic elements were isolated from *L. plumulosus*. One element, *hopper*, was activated by near lethal concentrations of TNT, phenanthrene, and lead. Activation of transposable elements suggests a general response to environmental shock and may serve as markers for lethal effects. We sought to determine if transposition events occurred resulting in an increase in genomic *hopper* insertions. Sublethal levels of TNT increased *hopper* genomic copy numbers 3.2-fold. Increased transposition directly impacted genomic DNA structure as evidenced by correlation of amplified fragment length polymorphisms to increasing *hopper* copy number. Mobilization of retrotransposons in response to chemical stress provides *L. plumulosus* an opportunity for rapid genome remodeling and adaptation to an environmental stressor.

Exposure to TNT induced movement of *hopper* may result in unexpected genotoxic and mutational events. Lead exposure effects were observed on superoxide dismutase gene expression that could result in sensitization of *L. plumulosus* to subsequent TNT exposures. Since significant effects were observed at sublethal levels of TNT, phenanthrene and lead exposure, genetic assays are useful as tools in screening field-contaminated sediments. Our investigation of the toxicity, mechanisms of action, bioaccumulation, and mixture interactions for TNT will provide valuable information when extrapolating laboratory data within ecological risk assessments.

5. PROJECT ACCOMPLISHMENTS

5.a PUBLISHED STUDIES

5.a.1 PUBLISHED STUDY 1: Lotufo, G.R., Farrar, J.D., Inouye, L.S., Bridges, T.S., Ringelberg, D.B. 2001. Toxicity of sediment-associated nitroaromatic and nitrocylamine compounds to benthic invertebrates. *Environmental Toxicology and Chemistry* 20 (8): 1762-1771.

The toxicity of nitroaromatic (2,4-diaminonitrotoluene [2,4DANT] and 1,3,5-trinitrobenzene [TNB]) and ^{14}C-labeled cyclonitramine compounds (hexahydro-1,3,5-trinitro-1,3,5-triazine [RDX] and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine [HMX]) to the marine polychaete *Neanthes arenaceodentata* and the estuarine amphipod *Leptocheirus plumulosus* following 10- or 28-d exposures to spiked sediments was investigated. Organismal-level effects on survival, growth, and reproduction and cellular-level effects on apoptosis (programmed cell death) were evaluated. Because cyclonitramines have low affinity for sediment, overlying water was not exchanged in the RDX and HMX exposures. Nitroaromatics sorbed strongly to sediment, resulting in near complete resistance to solvent extraction. Cyclonitramines sorbed weakly to sediment, as more ^{14}C-activity was found in the overlying water than in the sediment at exposure termination. No significant decrease in survival or growth was observed with cyclonitramines at initial sediment concentrations as high as 1,000 µg/g. Survival was significantly affected by nitroaromatics at nominal sediment concentrations as low as 200 µg/g, with *L. plumulosus* being more sensitive than *N. arenaceodentata.* Growth was significantly decreased at sublethal concentrations of 2,4DANT for *N. arenaceodentata.* Reproduction, measured only with L. plumulosus, was significantly decreased only in the highest RDX treatment and also in the lower TNB treatment. However, no decrease was observed in higher concentrations of TNB. Body burden at exposure termination was below detection limit (1 µg/kg) for all compounds. Significant inhibition of apoptosis was not accompanied by significant decreases in growth or reproduction. Because of its critical function in many biological processes, alterations in this endpoint may result in adverse effects on the organism and could be used as an early indicator of toxicity.

5.a.2 PUBLISHED STUDY 2: Steevens J.A., Duke, B.M., Lotufo, G.R., Bridges, T.S. 2002. Toxicity of the explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in sediments to *Chironomus tentans* and *Hyalella azteca*: Low-dose hormesis and high-dose mortality. *Environmental Toxicology and Chemistry* 21 (7): 1475-1482.

The toxicity of the explosives 2,4,6-trinitrotoluene (TNT); hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive [RDX]); and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high-melting explosive [HMX]), was evaluated in spiked sediment with two freshwater invertebrates. The midge *Chironomus tentans* and the amphipod *Hyalella azteca* demonstrated significant toxic effects after exposure to TNT and its degradation products, 1,3,5-trinitrobenzene (TNB) and 2,4-diamino-6-nitrotoluene (2,4DANT). Significant reductions in survival of *C. tentans* exposed to TNT, TNB, and 2,4DANT were observed at nominal sediment concentrations as low as 200 mg/kg. *Hyalella azteca* was more sensitive to TNT, TNB, and 2,4DANT than the midge, where significant reductions in survival were observed at nominal concentrations of 50, 100, and 200 mg/kg, respectively. Survival of the midge and the amphipod was unaffected after exposure to RDX or HMX at the highest concentrations of 1,000 and 400 mg/kg, respectively. Growth of the midge, measured as total weight, was significantly reduced by 2,4DANT. However, significantly increased growth was observed after exposure to sublethal concentrations of RDX and HMX. Although significant reductions in amphipod survival were observed at high concentrations of TNB, growth was significantly increased at sublethal concentrations. The results of the current investigation suggest that organisms exposed to explosives at contaminated sites may be affected at concentrations less than 25 mg/kg through hormetic growth enhancement and at higher concentrations through increased mortality.

5.a.3 PUBLISHED STUDY 3: Fredrickson, H., Perkins, E., Bridges, T., Tonucci, R., Fleming, J., Nagel, A., Diedrich, K., Mendez-Tenorio, A., Doktycz, M., Beattie, K. 2001. Towards environmental toxicogenomics: development of a flow-through, high-density DNA hybridization array and its application to ecotoxicity assessment. *The Science of the Total Environment*, 274:137-149

Assessment of the environmental hazard posed by soils / sediments containing low to moderate levels of contaminants using standard analytical chemical methods is uncertain due in part to a lack of information on contaminant bioavailability, the unknown interactive effects of contaminant mixtures, our inability to determine the species of a metal in an environmental matrix, and the relative sensitivity of bioassay species. Regulatory agencies compensate for this uncertainty by lowering cleanup goals, but in this process they effectively exclude otherwise attractive cleanup options i.e. bioremediation . Direct evaluations of soil and sediment toxicity preclude uncertainty from most of these sources. However, the time and cost of chronic toxicity tests limits their general application to higher levels of tiered toxicity assessments. Transcriptional level mRNA toxicity assessments offer great advantages in terms of speed, cost and sample throughput. These advantages are currently offset by questions about the environmental relevance of molecular level responses. To this end a flow-through, high-density DNA hybridization array genosensor system specifically designed for environmental risk assessment was developed. The genosensor is based on highly regular microchannel glass wafers to which gene probes are covalently bound at discrete 200-μm diameter spot and addressable 250-μm spot pitch locations. The flow-through design enables hybridization and washing times to be reduced from approximately 18 h to 20 min. The genosensor was configured so that DNA from 28 environmental samples can be simultaneously hybridized with up to 64 different gene probes. The standard microscopic slide format facilitates data capture with most automated array readers and, thus high sample throughput. In conclusion, hardware development for molecular analysis is enabling very tractable means for analyzing RNA and DNA. These developments have underscored the need for further developmental work in probe design software, and the need to relate transcriptional level data to whole-organism toxicity indicators.

5.a.4 PUBLISHED STUDY 4: Perkins, E., Lotufo, G. 2003. Playing in the mud- using gene expression to assess contaminant effects on sediment dwelling invertebrates. *Ecotoxicology* (in press).

Bioaccumulation and toxicity tests using benthic invertebrates such as the estuarine amphipod *Leptocheirus plumulosus* are typically used to assess the ecological risk associated with contaminated sediments. Despite their ecological and regulatory importance, little is known about such species at the genetic level. To begin understanding cellular and genetic responses of *L. plumulosus* to contaminants, we isolated several of their genes and developed quantitative assays to measure the effects of water exposures to 2,4,6-trinitrotoluene (TNT) and phenanthrene on gene expression. Real-time polymerase chain reaction (PCR) assays demonstrated that the expression of the genes for actin and a retrotransposon, *hopper*, was dependent on the exposure and tissue concentrations of those chemicals. Our data suggests that exposure to the explosive 2,4,6-trinitrotoluene and phenanthrene may induce movement of *hopper* resulting in unexpected genotoxic results.

5.a.5 PUBLISHED STUDY 5: Perkins, E., Lotufo, G., Farrar, J.D. 2003. Chemical stress induces transposition of a retrotransposon in a benthic amphipod (submitted to Nature Genetics, March 2003)

Transposable elements are thought to play a significant role in adaptation and evolution via insertions, deletions and nonhomologous recombination. Mobilization of transposable elements would permit rapid adaptive responses to environmental shocks. Yet, with the exception of plants and microbes, few examples exist where transposition occurs in response to chemical pollutants stress. Demonstration of transposition and subsequent genomic effects in response to contaminant exposure would provide significant support to the genomic shock theory. We have isolated several genes from *Leptocheirus plumulosus* similar to retrotransposons and transposons. *L. plumulosus* must adapt to many different chemicals as it feeds on sediment particles in estuaries from New England to Northern Florida in the United States. Transcripts of two non-LTR retrotransposons and two transposons accumulated with exposure to increasing amounts of TNT. *Hopper* expression also increased after exposure to phenanthrene, but not lead. We sought to determine if transposition events occurred resulting in an increase in genomic *hopper* insertions. Sublethal levels of TNT increased *hopper* genomic copy numbers 3.2-fold. Changes in amplified fragment length polymorphisms in exposed populations were correlated with increasing *hopper* copy number. Mobilization of retrotransposons in response to chemical stress provides *L. plumulosus* an opportunity for rapid genome remodeling and adaptation to an environmental stressor.

5.b. UNPUBLISHED STUDIES

5.b.1. UNPUBLISHED STUDY 1: Effect of 2,4,6-trinitrotoluene and lead exposure on gene expression in *Leptocheirus plumulosus.* Ed Perkins

Abstract:

We have developed ten rapid, quantitative gene expression assays for a widely used toxicity test organism, *Leptocheirus plumulosus*, to assess whether contaminants are biologically available to cause sublethal or lethal effects. The real-time polymerase chain reaction (PCR) assays were used to examine the effects of aqueous exposures of 2,4,6-trinitrotoluene (TNT) and lead on *Leptocheirus*. Expression of several genes were dependent upon aqueous and tissue concentrations. TNT caused a general increase in expression of target genes with the exception of a putative 26S protease regulatory subunit gene that decreased with sublethal exposure to TNT. Inhibition of 26S protease suggests that impaired cellular protein recycling may be a possible mechanism for hormetic effects previously observed in exposures to low concentrations of TNT. Lead accumulation resulted in decreased expression of a superoxide dismutase gene. The impact of lead on superoxide dismutase suggests that lead exposed *Leptocheirus* may be more susceptible to damage by oxidative toxicants such as TNT. These assays provide alternate sub-lethal measures for exposure effects and genotoxic potential in addition to contaminant availability to aquatic organisms. Gene-expression assays should prove useful in the screening of large numbers of sediment samples for potential for biological effects in lieu of costly and long-duration toxicity and bioaccumulation tests.

Introduction:

A realistic assessment of risk posed by contaminated sediment is obtained by determining contaminant bioavailability, exposure, and toxicity with ecologically relevant bioassay organisms. However current approaches are resource intensive and are limited to relatively few endpoints providing little insight into mechanisms of toxicity. Standard toxicity and bioaccumulation tests for sediment have several drawbacks. The tests are lengthy (weeks to months), expensive (thousands of dollars per sample), and typically have few end-points (survival, growth, and reproduction) (Gray *et al.* 1998). Rapid methods for screening large numbers of sediment samples containing moderate to low levels of chemicals are needed to discriminate between sites requiring active remediation from those where active remediation would be more detrimental than passive remediation. To better understand contaminant effects and mechanisms of toxicity in sediment bioassay organisms, we have studied genetic responses in *Leptocheirus* to aqueous contaminant exposures.

Current frameworks for evaluating sediment quality integrate toxicity and bioaccumulation testing (e.g., USEPA/USACE 1998). Several standardized exposure assays have been developed to assess toxicity of water and sediments using ecologically relevant species for freshwater, estuarine and marine habitats (Emery *et al.* 1997; USEPA/USACE 1998; USEPA 2000; DeWitt *et al.* 2001). In particular, the estuarine infaunal amphipod *Leptocheirus plumulosus* is used for both sediment and water testing. *Leptocheirus* is a burrow-builder that feeds on particles in suspension and on the sediment surface in estuaries from New England to Northern Florida (Bousfield 1973; DeWitt *et al.* 1992). *Leptocheirus* plays an important role as a food source in the Chesapeake Bay, where it can reach abundances of up to 29,000 M^{-2} (Holland *et al.* 1988).

Gene based assays promise speed, increased number of endpoints and an indication of how toxicants exert their effects. To develop these assays, genes that respond to exposure or are involved in critical biological functions must be isolated and characterized. Once genes are isolated and characterized, responses can be characterized using cDNA arrays or alternative methods such as semi-quantitative real-time reverse transcriptase PCR (SQRT-PCR). SQRT-PCR provides a rapid analysis of small numbers of genes (1-50) in large numbers of samples (Bustin 2000).

The objective of this study was to identify and isolate genes whose expression is influenced by exposure to chemical contaminants. Using aqueous exposures of a marine invertebrate to TNT and lead, we investigated whether these genes could provide insight into mechanisms of exposure, and whether these potential bioreporter genes are related to the onset of toxicity and the degree of bioaccumulation.

Materials and methods

Exposure media

A TNT spiking stock was prepared for each water treatment by combining ^{14}C-labeled TNT to non-radiolabeled TNT in the appropriate volume of acetone. Radiolabeled trinitrotoluene (^{14}C-TNT, 23.6 Ci mol^{-1}, 99% radiochemical purity) was purchased from New England Nuclear Research Products (Boston, MA). Non-radiolabeled TNT (99% purity) was purchased from Chem Service (Westchester, PA, USA). Exposure water was prepared by spiking 0.5 ml of TNT acetone stock to each liter of artificial seawater. The target radioactivity in all exposure water treatments was 5,000 dpm ml^{-1}. The target TNT concentration in water treatments were 0.25, 0.5, 1.0, 3, and 6 mg L^{-1}. For each treatment, measured specific activity was used for converting the radioactivity concentration in water or tissue samples to TNT molar-equivalent concentration, and therefore includes parent compound and breakdown or conjugated products. The control treatment consisted of a 0.5 ml L^{-1} acetone solution. For lead, exposure water solutions were prepared by adding the appropriate volume of a concentrated lead chloride aqueous (3,000 mg L^{-1}) solution to artificial seawater. The target lead concentrations in water treatments were 0.1, 0.2, 1.0, and 2 mg L^{-1}.

Aqueous exposures

Mature (3-8 mg) laboratory cultured *Leptocheirus plumulosus* (Emery *et al.* 1997) were used in all exposures. To identify genes affected by nitroaromatics and PAHs using differential display techniques, 100 amphipods were exposed to nominal concentrations of 2.5, 5, and 10 mg L^{-1} aqueous solutions of TNT or fluoranthene in beakers with 1 L water for 24 hours. At the end of the exposure period, amphipods were removed, and stored in a 3x volume of RNA-later (Ambion) at –0 ^{o}C until used for RNA isolation.

For 4-day dose-response exposures, 100 juvenile amphipods were exposed to a dose series of TNT or lead chloride in 1 L artificial seawater for 96 hrs. Amphipods were exposed in 4-L beakers. Four beakers were used for each treatment. Beakers were placed in a water bath at 23°C under gold fluorescent lights at a 16:h-8h light:dark cycle. Beakers were not aerated and no food was provided. The exposure solution from each beaker was fully renewed every 24 h. Exposure water was sampled for radioactivity determination (TNT) and chemical analysis (Pb) at the beginning of the experiment and daily thereafter before and after each exposure water renewal for monitoring compound concentration throughout the exposure period and to determined compound volatilization and degradation following the 24-h period preceding each exposure solution exchange event. One milliliter of water was transferred to 12-ml scintillation cocktail (3a70b; Research Products International, IL, USA) and ^{14}C-activity was quantified by liquid scintillation counting (LSC) on a Tricarb Liquid Scintillation Analyzer (Packard Instruments, Meridian, CT, USA). Measured mean concentrations were 0.38, 0.66, 1.39, 3.79, and 7.39 mg L^{-1} for TNT and 0.03, 0.12, 0.16, 0.74, and 1.51 mg L^{-1} for Pb. At exposure termination, 3-5 amphipods were blotted dry, weighed, and transferred to scintillation cocktail, and analyzed for ^{14}C-TNT radioactivity as described above. Remaining amphipods from each beaker were rinsed with water, blotted dry, weighed, pooled, and stored in a 3x volume of RNA-later (Ambion) at –80°C until used for chemical and genetic analysis.

RNA isolation.

Total RNA was isolated from 200 – 500 mg tissue (20-100 organisms) with an RNAgreen isolation kit using a mini-bead beater protocol and acid phenol extraction (Qbiogene) with the following modifications: after precipitation of RNA from aqueous solution with isopropanol precipitation solution two liquid phases were observed rather than pelleted RNA. The lower phase was removed after which 20 µl 12 M lithium chloride per 100 µl solution were added and RNA precipitated in a microfuge at 14 x g. RNA pellets were washed with 70% ethanol and suspended into 100 µl SAFE buffer. Contaminating DNA was removed using DNase and a DNA-free DNase removal kit (Ambion) as recommended by the supplier. RNA was quantitated using the RNA stain Ribogreen (Molecular Probes) as recommended by the manufacturer. RNA was stored at –20 ^{o}C until needed.

Isolation of conserved genes by consensus-degenerate PCR .

PCR primers used for isolation of conserved gene fragments are described in Fredrickson et al, (2001). Briefly, alignments present in the protein family database, Pfam (Bateman *et al*. 2002), were used as input to the program MAKEBLOCK and the consensus-degenerate primer design program, CODEHOP (Rose *et al*. 1998). Primers were checked for uniqueness against the GenBank sequence database using the GCG Wisconsin package program FindPatterns (accelrys). Genomic DNA from *Leptocheirus* was isolated from 200 mg tissue with FastDNA Kit (Qbiogene) using a mini-bead beater protocol. PCR reaction mixtures contained 75 ng of genomic DNA, 0.2 µM of dNTPs, 0.4 µM of primers, 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 40mM $MgCl_2$, 1 unit Taq DNA polymerase (Stratagene) in a total volume of 25 µl. The reaction mixture was heated 95ºC for 5 min and followed by 30 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1min. The final cycle was an extension at 72ºC for 10 min. PCR products were separated in a 20x20 cm, 7% non-denaturing Long Ranger^{tm} hydrolink gel (Cambrex). DNA was visualized after staining with SYBR Green I dye, using 310 nm transillumination and a ChemiImager 4000 CCD camera system (Alpha Innotech).

Cloning and sequencing of PCR fragments.

Bands were excised from gels using a razor blade or the tip of an 18G needle. PCR fragments were purified by heating the gel slice with 100 µl dH_20 at 100 ^oC for 5 min. The heated gel slice was removed by centrifugation and the PCR fragment purified from solution by precipitation with 1 µl Pellet Paint mussel glycogen co-precipitant (Novagen), 1/10 volume 3M Sodium Acetate (pH 5.2) and 3 volumes ethanol. Precipitated DNA was resuspended into 10 µl TE. 1 µl isolated PCR fragment was then reamplified using the primer set used in the original PCR reaction from which the band was isolated. Successful reamplifications were purified using a QIAquick PCR Purification Kit (Qiagen Corp.) according to manufacturer's protocols and cloned into the PCR fragment cloning vector pCR2.1-TOPO per manufacturers recommendations (Invitrogen). Plasmid DNA was isolated and cleaned using a QIAprep Spin Miniprep Kit (Qiagen). Clones were sequenced using an ABI 3100automated capillary DNA sequencer (Applied Biosystems) and Big Dye Terminator V. 3 fluorescent dye terminator cycle sequencing kits (Applied Biosystems) as recommended. Prior to sequencing, unincorporated dyes were removed using Centri-Sep purification columns (Princeton Separations). Resulting sequences were identified by comparison of all possible reading frames to known sequences in the National Center for Biotechnology Information's non-redundant protein database using the programs BLASTX with BEAUTY post-processing provided by the Human Genome Sequencing Center, Baylor College of Medicine (Worley et al 1998).

Gene expression analysis.

We designed real time PCR primers for genes having high similarity to 26S protease regulatory subunit, B4 (a gene of unknown function isolated by differential display), 60S ribosomal protein, superoxide dismutase I (SOD1), superoxide dismutase II (SOD2), and actin sequences using the software Primer Premier V. 4 (Premier Biosoft International). The primer sequences are: 26S (forward: TAACTTTAACGGTGCTGATT, reverse: AAACTTTCCTAACTGCTTTC), B4 (forward: GGCTAATCGTAACATGGGTG, reverse: ATTGGCTAGATTATTTCCCT), 60S (forward: AGAAGGCTGATGTAAGCGAGTT, reverse: GATACGGATGACGTGGAAGG), SOD1 (forward: TTTTCACAAGCCAGCACCAG, reverse: CGGCGAGTTTGAGCCATAA), SOD2 (forward: TTTTCACAAGCCAGCACCAG, reverse: CGGCGAGTTTGAGCCATAA), and actin (forward: GAGCGATGATCTTGATCTTGATG, reverse: TGTACCCAGGTATTGCTGACC). QuantumRNA universal 18S rRNA primers (Ambion) were used to determine abundance of 18S rRNA. We reverse transcribed 0.1 to 0.5 ug of total RNA into cDNA. 50 pmoles of random decamers were annealed to RNA in a 20 µl volume by heating to 85 ^{o}C for 3 minutes followed by chilling on ice. Reactions were begun with 10 µl of cDNA synthesis mix composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.4mM each deoxynucleotide triphosphate, 3 units RNase inhibitor, and 30 units M-MLV reverse transcriptase (Ambion). Reactions were placed at 42^{o}C for 2 hrs followed by 5 min at 95^{o}C to denature enzymes. Reactions were diluted to 200 µl prior to real time PCR assays. Real-time PCR assays were performed using an iCycler real-time PCR machine (BioRad). Five replicate reactions were performed for each treatment replicate. Real-time PCR reactions were composed of 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.0% Triton® X-100, 0.25 mM each deoxynucleotide triphosphate, SYBR Green I mix [final concentration of 5% dimethylsulfoxide, a 1/10,000 dilution of SYBR Green I (Molecular Probes), 1 mg ml^{-1} bovine serum albumin, 1% Tween® 20], 0.5 units Taq polymerase (Promega), 0.5 mM primers, and 2ul cDNA (representing approximately 2.5 ng total RNA) in a final volume of 20 µl. PCR conditions were 3 min at 95^{o}C, followed by 50 cycles of 95^{o}C for 30 sec, 55^{o}C for 45 sec, and 72^{o}C for1 min. Fluorescence of primer dimer products may overestimate abundance of desired PCR products. To minimize primer dimer interference, data were collected after extension at 72^{o}C during each cycle by insertion of a 10 second hold at a temperature, collection T_m, resulting in melting of primer dimers, but not desired products. Amplification of desired PCR products was determined using a melting curve from 55 ^{o}C to 95 ^{o}C in 0.4 ^{o}C increments.

Data analysis.

Threshold values of fluorescence (C_t) obtained for real time reactions were analyzed for differences between experimental organisms and control organisms by determining ratios of experimental to control C_t values using the following formula: target expression relative to control = (efficiency of amplification of target PCR product) $^{(\text{control average } C_t - \text{sample} C_t)}$. Expression ratios between samples were normalized to 18S rRNA as a measure of total RNA of samples relative to controls. Relative expression values were identified as outliers and excluded from analysis if they were in excess of one standard deviation from the mean. Students t-test was used to assess the significance of observed differences as implemented by Excel (Microsoft). Correlations of genetic data were performed using GraphPad Prism 3.0 (GraphPad Software Inc.). Statistical analysis of chemistry, survival and growth data was conducted using Sigma Stat version 2.03 (Jandel Scientific). Statistical analysis was conducted using nominal chemical concentration values due to difficulties associated with measuring explosives in sediment. Significant differences in survival and growth of organisms were detected using one-way analysis of variance (ANOVA) and comparisons between treatments were determined using Bonferroni's t-test. Where results did not have a normal distribution, ANOVA on ranks was used followed by Dunn's method for multiple comparisons. For the purposes of these experiments a value of $p < 0.05$ was used for testing of significance for all statistical tests.

Results

Isolation of conserved gene fragments from *Leptocheirus*.

A total of 22 different primer sets were designed from 6 different protein families including p450, DNA topoisomerase I, superoxide dismutase, glutathione-S transferase I, cyclin, and actin (Fredrickson *et al*. 2001). Using these primer sets 126 gene fragments from *Leptocheirus* were isolated. 34 clones had high similarity to known genes. Clones were isolated for actin and superoxide dismutase along with a probable pyruvate dehydrogenase and a clone with similarity to peptide ABC transporter protein (Table 5.b.1.A). Eighteen different clones were similar to conserved reverse transcriptase protein domains of retrotransposons and conserved transposase domains of transposons (Perkins et al, submitted). Most fragments characterized (92) had little similarity to known sequences.

Isolation of nitroaromatic and PAH responsive genes using differential display.

Several genes were successfully isolated from genomic DNA that are related to conserved genes with known functions. However the isolates have an unknown relationship to explosives toxicity, the focus of this work. To directly isolate genes responsive to military relevant compounds, *Leptocheirus* was exposed to a model explosive, TNT, and a model polyaromatic hydrocarbon, fluoranthene. Several cDNAs were isolated using differential display techniques. Three were further characterized by sequence analysis and semi-quantitative, reverse transcriptase, real time-PCR (SQRT-PCR) assays (Table 5.b.1.A).

Effects of TNT exposure on gene expression in *Leptocheirus*.

SQRT-PCR assays were developed to determine relative expression levels of six different genes isolated from *Leptocheirus* either by codehop primers or differential display (Table 5.b.1.A). We have also examined the effects of TNT on expression four transposable elements isolated from *Leptocheirus* (Perkins et al 2003, Appendix A). An additional assay to determine relative levels of 18S ribosomal RNA transcripts was used to normalize total RNA levels between assay and exposure replicates.

Exposure to TNT was measured as the total amount of bioaccumulated chemical (tissue residue) originally entering an animal in the form of TNT, expressed in terms of TNT equivalents or total radiolabeled compounds present in an organism post-exposure. Mortality was dramatically affected at exposures greater than 3.79 mg L^{-1} (Figure 5.b.1.A) or TNT body burden greater than 686 nmole g^{-1} tissue. Tissue residue levels appeared to increase linearly with exposure, although significant variation was observed among replicates.

All genes were affected by exposure to TNT (Figure 5.b.1.B). Effects appeared to reflect TNT equivalent tissue accumulation rather than exposure concentration. Expression of actin correlated (Spearman r = 0.8524, P (2-tailed) =0.0238) with TNT tissue residues below lethal levels (<500 nmole TNT equivalents g^{-1} tissue). We previously demonstrated that transcript levels of transposable elements hopper, tank, and stealth were also highly correlated (Spearman r = 0.9266, 0.08524, 0.9266 respectively and P (2-tailed) =0.0067, 0.0238, 0.0067 respectively) with TNT tissue residues (Perkins *et al*. 2003). QM protein, retrotransposon ranger and B4 increased at low-level doses of TNT (Figure 5.b.1.B). Expression of 26S protease regulatory protein was dramatically reduced at all doses of TNT. Superoxide dismutase gene assays failed to work with TNT exposed *Leptocheirus*.

Effects of lead exposure on gene expression in *Leptocheirus*.

Exposure of *Leptocheirus* to concentrations of lead from 0 to 1.51 mg L^{-1} resulted in proportional accumulation of lead in tissue from 0 to 750 mg kg^{-1} (Figure 5.b.1.C). A significant effect on survival was seen only at the highest exposure level (Figure 5.b.1.C). Lead had no significant effect on *hopper* and actin gene expression (Figures 5.b.1.D and E). Expression of the QM protein, b4, and the retrotransposon *hopper* increased only when survival was affected (Figures 5.b.1.D and E).

Sub-lethal effects were observed with expression of the oxidative stress detoxification enzyme superoxide dismutase, SOD2 (Figure 5.b.1.E). SOD2 expression levels were dramatically reduced at all concentrations. Expression levels inversely correlated with tissue levels (log SOD2 expression *vs*. lead tissue concentration r=-0.7197, n=13 and log SOD2 average expression to lead tissue concentration r=-0.8489, n=5).

SUMMARY:

We have isolated a number of genes from a common sediment bioassay organism, *Leptocheirus plumulosus,* including alpha-actin, Mn- SOD, probable pyruvate dehydrogenase E1 component, a gene similar to peptide ABC transporter binding protein, 26S protease regulatory subunit, a QM protein homologue, several mobile genetic elements, and genes of unknown function using conserved PCR in addition to differential display experiments. Quantitative gene expression assays were created for five genes, alpha-actin, Mn- superoxide dismutase, 26S protease regulatory subunit, QM protein, and for an unknown gene b4, in addition to four developed previously for transposable elements (Perkins *et al.* 2003). Gene expression of actin and the transposable elements *hopper*, *tank*, and *stealth* strongly correlated with sub-lethal bioaccumulation of TNT in 4-day exposures. Expression of QM, b4 and the retrotransposon *ranger* increased expression at very low doses of TNT, but generally decreased expression with high concentrations TNT. One gene, a probable 26S protease regulatory subunit, exhibited reduced expression at all doses of TNT.

The suppression of probable 26S protease regulatory subunit expression in concert with increased expression of other genes tested at low levels of TNT is consistent with previous observations of hormesis, or low-dose stimulatory effects, observed in exposures to explosives. Steevens *et al*. (2002) observed increased growth in two aquatic invertebrates in sediment exposures to RDX and TNB. Bailey *et al.*, (1985) observed an increase in offspring in *Daphnia magna* exposed to sublethal concentrations of TNT. The 26S protease regulatory subunit is one of the major pathways to degrade and recycle cellular proteins by ATP/ubiquitin-mediated proteolysis. Inhibition of this proteosome in *Drosophila* S2 cell lines and plants leads to programmed cell death and accumulation of polyubiquitinated proteins (Wojcik and DeMartino 2002; Kim *et al.*, 2003). By inhibiting 26S protease subunit expression, intracellular proteins could accumulate leading to apparent cell growth.

Lead exposure had no significant effect on actin or protease expression, but QM, b4, and *hopper* expression rose at concentrations causing lethal effects. SOD2 expression was directly related to sub-lethal lead tissue concentrations. SOD2 expression may be useful in quantitating bioavailability of lead in sediment. Reduction of superoxide dismutase expression suggests that lead exposure could sensitize organisms to further oxidative stress. We have cloned two different superoxide dismutase genes from *Leptocheirus,* SOD1 and SOD2, indicating the presence of a multigene family. While we have been unable to detect expression of SOD1, expression of alternate SOD genes could compensate for reduction in SOD2.

Although changes in gene expression caused by contaminant exposure relates to the onset of toxicity, basing determinations of toxicity solely on gene expression data from exposures at or past lethal concentrations may lead to false conclusions of lower toxicity. Further tests varying exposure period and with lower concentrations will determine how well effects in long-term (e.g., 28 d) exposures relate to effects seen on gene expression in short-term exposures.

5.b.2. UNPUBLISHED STUDY 2: Application of Population Modeling to Evaluate the Ecological Relevance of Toxicological Effects. Todd Bridges

Introduction

ASSESSING RISK TO POPULATIONS:

Assessing risks to abundant species commonly involves collecting data on toxicology effects on individual organisms and qualitatively extrapolating those effects to impacts on populations or communities of organisms in nature. An extrapolation is necessary because the primary focus of concern in ecological risk assessment is the viability of populations rather than the vitality of an individual organism within a population (Bridges *et al.*, 1996). Within the context of sediment risk assessments toxicity tests are used to assess the hazard posed by sediment. Individual organisms are exposed to the sediment of concern and individual responses in survival, growth and reproduction are observed. During the coarse of this project we have developed molecular methods for assessing toxicity in individual organisms to provide more specific information about the effects observed in less time than can be achieved using current approaches with bioassays and whole organisms. However, using either molecular or whole organism responses to a contaminant stress to reach conclusions about risk requires developing a logic that relates these responses to population viability. To provide a more robust means of linking measures of toxicity with population-level impacts we developed population models for experimental organisms used in our study.

USE OF MATRIX POPULATION MODELS:

Matrix models have been commonly applied in the field of ecology to describe population dynamics for more than 25 years (Caswell, 1989). Specifically, these models are applied for population projection when survival rates and birth rates are thought to depend on age or developmental stage. Using matrix models the population at any time-step is represented as a vector of age-specific or stage-specific population sizes. Multiplying this vector by the transition matrix yields the population size at the next time-step. This is shown in equation 1 (appendix B) for a general age-based transition matrix with a total of m age classes, where $n_i(t)$ refers to the number of individuals in age-class i at time t, F_i represents the fecundity, or birth-rate, of individuals in age-class i, and S_i refers to the survival rate of individuals in age-class i. The width of each age-class, except the last (the m^{th} age-class), is equal to one time-step. For example, if we use one-week age classes (0-1 weeks, 1-2 weeks, etc.), then multiplication by the transition matrix would represent a change in the population over one week in time. The last age-class is known as a composite class, defined as all individuals older than a certain age.

After several applications of the transition matrix, the population approaches a stable age distribution, in which the proportion of individuals in each age-class remains constant between successive time-steps. At this point, the population grows (or declines) exponentially at a rate determined by the largest eigen-value of the matrix, as shown in equation 2 (appendix B).

In this equation, N_t represents the total population size at time t, and λ (lambda) represents the largest eigen-value of the transition matrix. Lambda, also referred to as the finite rate of increase, is generally considered an appropriate summary statistic from which to infer the relative status of a population. At the stable age distribution, if lambda is greater than one, the population will increase exponentially, whereas if lambda is less than one, the population will experience exponential decline to extinction. The major criticism of the use of lambda as a measure of population growth is that natural populations are rarely observed to be at the stable age distribution for a measured transition matrix. This is often due to the fact that the survival rates and fecundities, which comprise the matrix, do not remain constant through time. However, relative values of lambda and the change in lambda can be very useful in summarizing the effects of a particular factor on population growth.

Materials and Methods

APPLICATION TO *Leptocheirus*:

We applied matrix population modeling to *Leptocheirus* using effects data collected following exposure to three dilutions of Black Rock Harbor sediment (BRH) (e.g., Bridges and Farrar, 1997). Toxicity was measured in each of the three dilutions of BRH under two food rations to evaluate nutritional effects on toxicity. Three different BRH concentrations were used (0%, 3%, and 6% BRH) and two levels of food ration were applied (normal food ration, 1x; and double the normal food ration, 2x). Survival and reproductive responses were measured in these six treatments (0%-1x, 0%-2x, 3%-1x, 3%-2x, 6%-1x, and 6%-2x) over a period of 30 weeks (Figure 5.b.2.A). These data were then used to parameterize an age-classified matrix population model for *Leptocheirus*.

Survival:

The survival rate of age class $\underline{x}$ individuals is defined as the probability of an individual in age class $\underline{x}$ reaching age class $\underline{x}+1$ after one time unit has passed. Since the animals in the experiments were within one week of each other in age, the best estimate we have of this probability (where the time unit is defined as one week) is the number of individuals counted at week $\underline{x}+1$ divided by the number of individuals counted at week $\underline{x}$. We considered using the birth-flow formula (Caswell, 1989), but one necessary element of that formula is $\underline{l(x)}$, or the probability of surviving from birth to exact age $\underline{x}$. Since we did not have animals that were exactly the same age (we cannot even say that they were within one day in age, only that they were within one week in age), we could not reliably estimate $\underline{l(x)}$ for any age. Therefore, we could not use the birth-flow formula, and instead estimated the survival rate as described above. This is characterized in equation 3 (appendix B). In this equation, $\underline{count(x)}$ simply refers to the number of individuals counted at week $\underline{x}$. Age class $\underline{x}$ is defined as individuals between $\underline{x}$ and $\underline{x}+1$ weeks old.

Fecundity:

The fecundity of age-class x individuals, f_x, is defined as the number of age class 0 individuals alive at time t+1 per age class x individual at time t. Again, we could not use the birth-flow formulas since we did not have reliable estimates of $l(x)$ (neither did we have reliable estimates of $m(x)$, or the expected number of offspring per time unit of individuals aged x) since we did not have data on individuals of exactly the same age. Thus we calculated f_x as the number of offspring counted at week x+1 divided by the number of individuals counted at week x. This is characterized in equation 4 (appendix B).

Age class width and degree of compositing:

In defining the transition matrix, we used one-week age classes and thus a one-week time step. We pooled individuals aged nineteen weeks and older, for a total of twenty age classes. The survival rate of the composite age class was calculated as the weighted average of the week-specific survival rates of the age-classes comprising the composite class, weighted by the number of individuals in each age class. Similarly, the fecundity of the composite class was calculated as the weighted average of the week-specific fecundities of the classes comprising the composite class.

Calculating contributions:

After defining transition matrices for the six different treatments, we designated one treatment as the control and one treatment as the experimental for nine combinations of control and experimental. The change in lambda is defined as lambda for the experimental treatment minus lambda for the control treatment (see appendix B, equation 5). For each combination of experimental and control there was an expected direction of change of lambda, depending on which factor was varied.

The change in lambda can be broken down, or decomposed, into the sum of contributions from the survival rates and fecundities, by use of equation 6 (appendix B; Caswell, 1989). The partial derivative of lambda with respect to a given vital rate is evaluated at the arithmetic mean of the control matrix and experimental matrix. Each element in the summation is then the absolute change in a given vital rate multiplied by the partial derivative of lambda with respect to that vital rate, which is approximately how much of the change in lambda is due to the change in that vital rate. We will refer to this as the contribution by that vital rate (the ij^{th} vital rate) to the change in lambda. Note that this is a first-order, and thus a local approximation; for large changes in lambda, the approximation is likely to break down.

Significance tests:

An ANOVA was performed, using SPSS software, to test for the effects of each factor on lambda and their interaction. However, to test for significance of the contribution of each matrix element, an ANOVA could not be employed because two treatments must be designated in order to define any given contribution. Thus for each combination of control and experimental treatments, bootstrapping was performed to test for the significance of the contribution of each vital rate. This was done by randomly selecting (with replacement) six replicates from the six replicates of both the control and experimental treatment, calculating the contribution of each vital rate, and comparing this sampled contribution with zero. We are comparing with zero because the null hypothesis is that there is no contribution for any given vital rate, i.e. that the contribution is zero. The test for significance was one-tailed, since we had *a priori* reasons to believe that the change in lambda should be in a particular direction. For example, when the experimental treatment had more sediment than the control, we had reason to believe that lambda would decrease, simply because the sediment is known to contain pollutants. On the other hand, when the experimental treatment had more food than the control, lambda was expected to increase.

Results and Discussion

From Table 5.b.2.A one can see that increasing sediment concentration decreased lambda, while increasing food ration increased lambda, as expected. Analysis of variance revealed that there was a highly significant effect of sediment concentration on lambda ($p<0.001$), while food ration had a marginally significant effect on lambda ($p=0.036$), and the interaction between food and sediment was non-significant. From this statistical analysis, we can conclude that BRH had a significant adverse effect on the population growth of *Leptocheirus*.

As there were six different treatments applied in the lab to *Leptocheirus*, we were able to define several control/experimental treatment combinations. For each combination, only one of the two factors was varied. The combinations and the change in lambda for each are shown in Table 4.

From Table 5.b.2.B one can see that the change in lambda was always in the expected direction: when BRH concentration was increased, lambda decreased, and when food ration was increased, lambda increased. These results would be expected primarily because the sediment was considered toxic and an increase in food ration will cause an increase in the rate of population growth.

DECOMPOSING THE CHANGE IN LAMBDA FOR *Leptocheirus*:

As stated earlier, an analysis of variance revealed that sediment had a highly significant effect on lambda ($p<0.001$), and food had a marginally significant effect on lambda ($p=0.036$). The next question is whether this change in lambda is due to changes in survival rates or fecundities or both, and which vital rates in particular caused lambda to change the most. To address these questions, the change in lambda was decomposed into contributions from the various survival rates and fecundities for each control/treatment combination. For example, consider the combination where the control treatment is 0%-1x, i.e. 0% BRH sediment (control sediment) and normal food ration, and the experimental treatment is 6%-1x, or 6% sediment and normal food ration. Since there was an increase in BRH concentration, but food was held constant, we expected lambda to decrease, which is in fact what occurred: lambda decreased from 1.543 down to 1.322, for a change in lambda of -0.221. Figures 5.b.2.B and 5.b.2.C show that the largest (negative) contributions to the change in lambda for this combination came from the survival rate of individuals approximately one week old and the fecundity of individuals approximately five weeks old (both affected a change in lambda of approximately -0.045).

SUMMARY OF RESULTS:

The largest contributions to the change in lambda for *Leptocheirus* were generally due to changes in survival rates of age classes 1 through 3 and fecundities of age classes 4 through 8. In all cases, the overall change in lambda was in the expected direction; when sediment concentration was increased from control to experimental, lambda decreased; when food was increased from control to experimental, lambda increased. The largest contributions (in the expected direction) were generally statistically significant (statistical significance of contributions was checked by bootstrapping), and several smaller contributions, from older age classes, also proved to be statistically significant.

These experimental results suggest that the 28-day duration of the *Leptocheirus* chronic sediment toxicity test should be sufficient to capture effects on survival and reproduction with the greatest influence on population growth. The decomposition analysis has shown that the main effects on *Leptocheirus* population growth come early in life, through effects on the survival (between one and four weeks old) and reproductive (between four and nine weeks old) rates of young individuals.

RISK APPLICATIONS:

Populations models and decomposition analyses provide a mechanism for relating effects observed at the sub or whole organism level to potential impact on populations. Such tools are critical to the process of evaluating the ecological relevance of toxicity data and to developing regulatory acceptance for the use of sub or whole organism toxicity results in ecological risk assessment.

5.b.3. UNPUBLISHED STUDY 3: Comparative and mixture toxicity of TNT and TNT daughter compounds to a freshwater midge in aqueous exposures. Gui Lotufo.

INTRODUCTION

Nitroaromatic compounds are commonly found in the environment (e.g., soil, sediment, ground water) as complex mixtures. TNT is one the most widely used munitions in the world. Because of its toxicity to plants, microbes and animals, contamination of soils and sediments by TNT poses a potentially serious environmental and public health problem around military facilities. In addition, TNT typically co-occurs with its transformation products as a result of multiple chemical and biological transformations in water, soil and sediments. Degradation products of TNT are formed through reduction and oxidation pathways. Reduction of TNT occurs through amination of the nitro groups on the ring, initially forming aminodinitrotoluenes (2- or 4ANDT), and subsequently diaminonitrotoluene (2,4- or 2,6DANT) and lastly triaminonitrotoluenes. Photooxidation of TNT forms trinitrobenzoldehyde. Subsequent decarboxylation occurs under aerobic conditions and results in the formation of trinitrobenzene (TNB). In addition, TNB has been manufactured as an explosive and for other military applications. Individual nitroaromatic compounds may not be present at concentrations that result in adverse biological effects. However, joint exposure to the components of a nitroaromatic mixture may result in adverse toxicological effects. Compounds in a mixture may interact additively, independently, synergistically, or antagonistically. The current research is being conducted to compare single-compound toxicity and evaluate the nature of chemical interactions of TNT and representatives of its daughter compounds in aqueous exposures.

MATERIALS AND METHODS

Experimental organisms

Chironomus tentans were obtained as egg masses from Environmental Consulting and Testing (Superior, WI). Upon arrival to the laboratory, egg masses were placed in plastic tote tubs containing dechlorinated water and a thin layer of sand. Fish food flakes (Tetrafin®, Tetra Sales) were added daily. Larvae were reared for 10 or 11 days after hatching to the third instar based on a head capsule width ranging from 0.33 to 0.45 mm (USEPA, 2000).

Chemicals

The following compounds were used in the experiments: 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), 2-amino-4, 6-dinitrotoluene (2ADNT), and 2,4-diamino-6-nitrotoluene (2,4DANT). TNT was purchased from Chem Service (Westchester, PA), TNB was purchased from Supelco (Bellefonte, PA), 2ADNT and 2,4DANT were purchased from SRI International (Menlo Park, CA). Purity was >98% for all compounds.

Aqueous toxicity experiments

The toxicity of TNT, TNB, 2ADNT and 2,4DANT was determined in 10-d single-compound exposures to aqueous solutions. Five concentrations, selected using results from range-finding experiments, were used for each compound (Table 5.b.3.A). The toxicity of a mixture of TNT, TNB, 2ADNT and 2,4DANT was also examined in 10-d exposures to aqueous solutions. Mixture treatments consisted of five equimolar mixtures of all nitroaromatic compounds (Table 5.b.3.B). Therefore, the target concentration of each individual compound in the mixture was one-fifth the target sum concentration (Table 5.b.3.B).

Nitroaromatic compounds dissolved in acetone were added to dechlorinated tap water (1.6 ml acetone /L) for preparing aqueous exposure solutions. Exposure chambers were 300-ml beakers filled with 250 ml of exposure solution and 5 g of quartz sand as a substrate for tube building by the midges. Each beaker received 10 larval midges. Four replicates were used for each treatment. Beakers were placed in a water bath at 23°C and received trickle flow aeration under gold fluorescent lights at a 16:h-8h light:dark cycle. Approximately 95% of the exposure solution from each beaker was renewed every 24 h and food was provided every day as 6 mg of TetraFin. Fresh aqueous solutions were prepared before each water exchange. Aqueous solutions were sampled on days 0 and 6 of the experiment. Exposure water was sampled from one beaker per treatment before water exchange on days 1 and 7 of the experiment for assessing compound volatilization and degradation following the 24-h period preceding each exposure solution exchange event. Temperature and dissolved oxygen were monitored throughout the ten-day exposure period. Hardness, alkalinity, and ammonia concentration were measured using commercially available kits (LaMotte, Charleston, SC) at initiation and completion of the experiments. Temperature and dissolved oxygen were monitored throughout the ten-day exposure period. Water quality parameters including hardness, alkalinity, and ammonia were also measured using a commercially available kit from Lamotte (Chestertown, MD) at the initiation and completion of the exposures. Water quality parameters were within acceptable limits throughout the experiment: dissolved oxygen (6.5-6.9 mg/l), pH (7.4-7.6), ammonia (< 3 mg/L), hardness (100-108 ppm $CaCO_3$), alkalinity (104-116 ppm $CaCO_3$). At experiment termination, all midges from each beaker were rinsed with water, blotted dry, weighed, and frozen at –80°C for chemical analysis of nitroaromatic compounds in tissues. Therefore, biomass, expressed as wet weight, was determined for the pool of midges recovered from each exposure beaker.

Chemical analysis

Water samples. Aqueous samples were analyzed for TNT and its daughter compounds animodinitrotuenes (2ADNT and 4ADNT) and diaminonitrotoluenes (2,4DANT and 2,6DANTs) using a modified version of U.S.EPA method 8330. Chemical analysis for TNT and degradation products in water was conducted using an Agilent 1100 HPLC (Palo Alto, CA, USA) equipped with a diode-array detector (USEPA 1997). The column used was a SUPELCO RP-Amide C-16 with a sample injection volume of 100 µL and flow rate of 1 ml/min. Solvent ratios were 45% water and 55% methanol and UV absorbance was measured at 230 nm. Laboratory reporting limits for the analysis of water samples using this method are 0.1 ppm for TNT, TNB, 2ADNT, 4ADNT, and 2,4DANT.

Tissue samples. To ensure sufficient biomass for solvent extraction, replicate samples from each treatment were combined and transferred to polypropylene bead-beater vials. Sample mass ranged from 60 to 270 mg wet wt. Each vial received 100 mg of 0.5-mm glass beads and 0.75 ml of HPLC grade acetonitrile. Samples were homogenized on a mini beadbeater (Biospec, Barttlesville, OK) for 100 sec at 4200 oscillations/min and placed on ice. Samples received 0.75 ml of 1% $CaCl_2$ and were sonicated (Branson 3200, Branson Ultrasonics, Danbury, CT) for 1 h at 18°C in a water bath (Neslab RTE-111, Neslab Instruments, Newington, NY). Samples were centrifuged for 10 min at 7500 x g at 4°C. The supernatants were filtered through 0.45 µm PTFE syringe filters (Nalge Nunc, Rochester, NY) into amber sample vials. Analytes were isolated and identified as described for water samples.

Calculations and statistical analysis

All measurement values are expressed as a mean ± 1 standard deviation. Completely randomized one-way analysis of variance was used to compare survival and growth data. William's test was used for comparing treatment means with control means. Significance level (a) was set at 0.05. Survival data were transformed by arcsine-square-root before analysis. Median lethal concentration (LC50) values were calculated using the trimmed Spearman-Karber method.

For each mixture treatment, the toxic unit for each compound in the mixture was calculated by dividing its concentration by the LC50 derived from the single-compound exposure conducted concomitantly with the mixture experiment. For each mixture treatment, total toxic units (STU) were calculated as the sum of the individual compounds toxic units.

RESULTS

Exposure water concentrations

Substantial degradation of TNT and TNB occurred during the 24-h between exposure water exchanges (Table 5.b.3.A). TNT degraded mostly to 4ADNT but DANTs were also detected; the fraction of breakdown products relative to the sum molar concentration of all compounds was high across treatments. TNB degraded to 1-amino-3,5-dinitrobenzene (ADNB); the degree of degradation decreased with increasing target concentration. The breakdown product 2,4DANT was also found in the 2ADNT aqueous solutions, but the degree of degradation was lower than those observed for TNT and TNB. Determination of sum molar concentration for all compounds detected in the exposure solution during the period between water exchanges allowed the calculation of the percent loss of compound over 24-h periods. Compound losses were greater in the TNT and TNB experiments and lowest for the 2,4DANT experiments. Mean water concentrations for each treatment (Table 5.b.3.A) were the mean for the average sum molar concentration of all quantifiable compounds measured before and after water exchanges at days 0-1 and 6-7. Mean water concentrations were typically lower than target concentration, with differences being greater for compounds with the highest percent loss between water exchange events (Table 5.b.3.A).

Comparative Toxicity

Survival was high in the control (95±10%) and tended to decrease with increasing concentrations for all nitroaromatic compounds (Fig. 5.b.3.A). Aminated compounds (2ADNT and 2,4DANT) were less toxic than non-aminated compounds (TNT and TNB), as indicated by the LC50 calculated for each compound (Table 5.b.3.C). Midges were substantially more tolerant to the most aminated compound (2,4DANT) than to any other compound, as the range of lethal concentrations was over one order of magnitude higher for 2,4DANT than for the other compounds (Fig. 5.b.3.A). The relative toxicity of nitroaromatic compounds in relation to the toxicity of TNT, as indicated by the ratio between their LC50s was 1:1.2 for TNB, 1:2 for 2ADNT, 1:24 for 2,4DANT. Significant reductions in growth were observed for TNT, 2ADNT, 2,4DANT (Fig. 5.b.3.A). Decreases in growth occurred at concentrations where survival was not significantly, and therefore, such decreases were not sublethal responses to aqueous exposures to nitroaromatics.

Mixture interaction

The sum molar LC50 for the mixture exposure was 15.1 µmol/L, therefore within the range of the LC50 for the single compounds excluding 2,4DANT. Survival decreased with increasing STU (Fig. 5.b.3.B), with significant mortality occurring at STU of 1.5 and higher. The sum toxic units for each treatment and the survival data were used in the calculation of a sum toxic unit LC50, which is expected to equal 1 when the compounds in the mixture interact in an additive manner, i.e., without decreasing (antagonism) or enhancing (synergism) the overall response. The sum toxic unit LC50 was 0.95 (95% CI = 0.80 – 1.13) for the nitroaromatic mixture, strongly suggesting response additivity of mixtures of nitroaromatic compounds.

Bioaccumulation and critical body residues

Nitroaromatic compounds were detected in solvent extracts of live midges from all experiments, except TNB, and body residues for those treatments with (Table 5.b.3.D). Organisms exposed to TNT accumulated ADNTs; those exposed to 2ADNT accumulated mostly the parent compound but also some 2,4DANT. The relationships between body residues, expressed as the sum molar concentrations of nitroaromatic compounds, and significant effects in survival are presented in Table 5.b.3.D and Fig. 5.b.3.C. Across compounds, critical body residues ranged from 4.8 to 111.3 µmol/kg and appeared to be lower for exposures to 2ADNT and TNT and highest for exposure to 2,4DANT. Bioconcentration factors were calculated as the ratio between the sum molar concentrations in the tissue at exposure concentration to the mean water concentration for each treatment (Table 5.b.3.D). Overall, BCF values were highest for mixture treatments and lowest for 2,4DANT (Fig. 5.b.3.D).

DISCUSSION

In this investigation we compared the relative toxicity of TNT and three of its breakdown products to a freshwater invertebrate. TNB were similarly toxic to TNT. Nitro-reduction substantially decreases the toxicity of TNT, as the LC50 for the mono-aminated compound 2ADNT was higher than that for TNT by a factor of 2.2. Further amination appears to decrease toxicity even more dramatically, as the LC50 for the di-aminated breakdown product 2,4DANT was 24 times higher than that for TNT (Table 5.b.3.C). In a comparative toxicity study conducted in our laboratory (Houston and Steevens, unpublished), the aqueous toxicity of TNT and its breakdown products were compared using the freshwater amphipod *Hyalella azteca*. As observed with *C. tentans*, TNT, TNB and 2ADNT were similarly toxic to the amphipod and the LC50 values for these compounds (16, 6.7, and 19.4 µmol/L, respectively) were remarkably similar to those derived for *C. tentans*. However, 2,4DANT was more toxic than TNT for *H. azteca*, as indicated by its LC50 (11 µmol/L), a value substantially lower than the 2,4DANT LC50 for *C. tentans*. In a comparative study with the cladoceran *Ceriodaphnia dubia*, Griest *et al*. (1998), a yet different pattern of relative toxicity was reported. For *C. dubia*, both 2,4DANT (LC50 = 0.14 µmol/L) and TNB (LC50 = 3.8 µmol/L) were substantially more toxic than TNT (LC50 > 26 µmol/L) whereas 2ADNT (LC50 = 25 µmol/L) was similarly toxic to TNT. Therefore, whereas the toxicity of TNT was similar for the three species of invertebrates discussed above, the relative toxicity TNT breakdown products among themselves and relative to TNT is clearly species-specific. Interacting chemicals result in toxicological effects or responses that are difficult to predict based upon single chemical toxicological data. These interactions are defined as additive (summation of toxic responses from multiple chemicals in proportion to the concentration of each chemical in the mixture; each chemical contributes to the resulting response or effect without modifying the mechanism or effect of other chemicals in the mixture), synergistic (toxic response is greater than would be predicted by additivity; chemicals enhance the toxicological effect of other chemicals) or antagonistic (toxic response is less than would be predicted by additivity; chemicals decrease the toxicological effect of other chemicals). Results from this investigation clearly demonstrate that TNT and its daughter compounds interact additively when in a mixture. Therefore, the toxicity of a mixture to a given receptor can be reasonably predicted using single-compound toxicity derived for that receptor. However, comprehensive explosive compounds toxicity data sets are not available for aquatic receptors. Although aquatic species appear to be similarly sensitive to TNT in aqueous exposures, toxicity to TNT breakdown products should be considered highly species-specific and generation of receptor-specific toxicity data for risk assessment is recommended.

5.b.4. UNPUBLISHED STUDY 4: Comparative and mixture toxicity of sediment-associated TNT and TNT daughter compounds to a freshwater midge. Gui Lotufo**.**

INTRODUCTION

TNT typically undergo breakdown when associated with soils and sediments, either in laboratory (Lotufo *et al.* 2001; Steevens *et al.* 2002, see appendix A)) or field conditions (Rosser *et al.* 2001). Therefore, TNT and its daughter compounds are commonly found in the environment as complex mixtures. Individual nitroaromatic compounds may not be present at concentrations that result in adverse biological effects. However, joint exposure to the components of a nitroaromatic mixture may result in adverse toxicological effects. Compounds in a mixture may interact additively, independently, synergistically, or antagonistically. The current research is being conducted to compare the single-compound toxicity and evaluate the nature of chemical interaction of TNT and representatives of its daughter compounds in sediment exposures.

MATERIALS AND METHODS

Experimental organisms

Chironomus tentans were obtained as egg masses from Environmental Consulting and Testing (Superior, WI). Upon arrival to the laboratory, egg masses were placed in plastic tote tubs containing dechlorinated water and a thin layer of sand. Fish food flakes (Tetrafin®, Tetra Sales) were added daily. Larvae were reared for 10 or 11 days after hatching to the third instar based on a head capsule width ranging from 0.33 to 0.45 mm (USEPA, 2000).

Chemicals

The following compounds were used in the experiments: 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), 2-amino-4,6-dinitrotoluene (2ADNT), and 2,4-diamino-6-nitrotoluene (2,4DANT). TNT was purchased from Chem Service (Westchester, PA), TNB was purchased from Supelco (Bellefonte, PA), 2ADNT and 2,4DANT were purchased from SRI International (Menlo Park, CA). Purity was >98% for all compounds.

Comparative toxicity experiments

The toxicity of TNT, TNB, 2ADNT and 2,4DANT was determined in 10-d single-compound exposures to sediments spiked at different concentrations (Table 5.b.4.A). Nitroaromatic compounds were dissolved in 5 ml of acetone for preparing spiking solutions. Nitroaromatic compounds were added to clean sediment (Browns Lake, Engineering Research and Development Center, Vicksburg, MS, pre-sieved < 300 µm, solids content = 59%, total organic carbon 1.3%) by slowly adding dosing solutions to homogenized sediment (350 g) with constant mixing using a stir plate in 1-L glass beakers. Control sediments received 5 ml of acetone. After mixing for 1 hour under gold fluorescent light, spiked sediments were immediately added to exposure samples to avoid excessive degradation of the toxicants. Each exposure chamber (300 ml tall beaker) received 75 g of sediment and 175 ml of dechlorinated water. Four replicates were used for each treatment. Beakers were placed in a water bath at 23°C and received trickle flow aeration under gold fluorescent lights at a 16:h-h light:dark cycle. Overlying water in the beakers was renewed every 12 h using an Envrion Tox® flow through bioassay diluter (Easley, SC). Midges in each beaker were fed daily with 6 mg of TetraFin. Temperature and dissolved oxygen were monitored throughout the ten-day exposure period. Hardness, alkalinity, and ammonia concentration were measured using commercially available kits (LaMotte, Charleston, SC) at initiation and completion of the experiments. Experiments with all four compounds were conducted simultaneously and shared a single set of control beakers. At experiment termination, the overlying water was removed avoiding disturbing the sediment, the sediment gently homogenized and approximately 5 g was removed for chemical analysis. The remaining sediment was sieved to remove all midges from each beaker. Surviving midges were enumerated, rinsed with deionized water, blotted dry, and weighed for wet biomass determination.

Mixture toxicity experiments

For assessment of mixture effects, a sum toxic unit (TU) approach was used (Marking, 1977). Simple additivity of TNT and TNT daughter compounds was assumed as a null hypothesis. The TU is the ratio of the compound concentration and its LC50 when present alone. The TU of a mixture is calculated by summing the individual TU of all compounds in the mixture. Mixture treatments were comprised of equipotent concentrations of TNT, TNB, 2ADNT and 2,4DANT targeting sum TU (STU) for the mixture ranging between 0.25 and 3 (Table 5.b.4.B). The toxic unit approach was used for defining the concentration of each compound in each treatment of the sediment mixture experiment. Each treatment corresponded to a target sum STU where each of 4 compounds was expected to contribute 25% of the sum. Concentrations of single compounds in the sediment mixture treatment were one-forth the product of the target STU and the compound LC50. As an example, the treatment targeting a sum TU of 1 was prepared by adding each compound at target concentration corresponding to one-forth its LC50. Ten-day LC50 values calculated for the comparative toxicity experiment using expected sediment concentrations were used in the calculations of single compound concentrations in the mixtures treatments.

Ten-day single-compound sediment exposures were conducted simultaneously with the mixture experiment for all individual nitroaromatic compounds used in the mixture (Table 5.b.4.C) to generate single-compound LC50 for the calculation of measured TU for the mixture treatments. For the mixture and single-compound experiments, experiment set-up and exposure conditions were as those described for the comparative toxicity experiments. Single-compound and mixture experiments were conducted simultaneously and shared a single set of control beakers. At experiment termination, surviving midges were enumerated, rinsed with deionized water, blotted dry, and weighed for wet biomass determination.

Chemical analysis

Spiking solutions. To obtain an estimate of the total amount of compound spiked to each sediment treatment, the total amount of compound in each dosing solution was estimated. A known volume of each dosing solution was diluted in acetonitrile to create solutions ranging from 5 to 30 µg/ml. Each diluted solution was further diluted in nanopure water (50:50, v:v). Analytes in the diluted dosing solutions were isolated and quantified and separated and by HPLC. The column used was a SUPELCO RP-Amide C-16 injecting samples at a flow rate of 1 ml/min with a 45% water 55% methanol solvent and UV absorbance measured at 254 and 230 nm.

Sediment. Sediment samples (5 g, wet weight) were vigorously mixed with 10 ml of acetonitrile and sonicated for 18 h (Branson 3200, Branson Ultrasonics, Danbury, CT) at 18°C in a water bath (Neslab RTE-111, Neslab Instruments, Newington, NY). The sediment was not air-dried before extraction because this procedure has been demonstrated to decrease the solvent-extractability of TNT and its daughter compounds (Lotufo, umplublished). Five ml of the extractant was recovered, filtered through PTFE syringe filters (Nalge Nunc, Rochester, NY) and an equal volume of water. Analytes in the extract were separated and quantified as described above. Sediment moisture content was determined by the weight ratio of wet and oven dried (55°C) triplicate samples.

Calculations and statistical analysis

All measurement values are expressed as a mean ± 1 standard deviation. Completely randomized one-way analysis of variance was used to compare survival and growth data. William's test was used for comparing treatment means with control means. Significance level (a) was set at 0.05. Survival data were transformed by arcsine-square-root before analysis. Median lethal concentration (LC50) values were calculated using the trimmed Spearman-Karber method.

For each mixture treatment, the toxic unit for each compound in the mixture was calculated by dividing its concentration by the LC50 derived from the single-compound exposure conducted concomitantly with the mixture experiment. For each mixture treatment, total toxic units (STU) were calculated as the sum of the individual compounds toxic units. Ten-d LC50 values, expressed as TU, were calculated using the STU from each treatment and survival data from individual replicates.

RESULTS

Comparative toxicity experiments

Expected concentrations in spiked sediments (Table 5.b.4.A) were calculated by dividing the total amount of compound spiked, estimated from the chemical analysis of dosing solutions by the sediment estimated dry weight. Expected concentrations approximated the target concentrations in all experiments. Measured sediment concentrations (Table 5.b.4.A) were determined by chemical analysis of solvent extracts of sediment samples taken immediately following spiking and mixing.

Measured initial concentrations of all compounds were typically lower than expected concentrations (Table 5.b.4.A), this was most pronounced for the lower treatments. This difference indicates that a fraction of the spiked compound resisted solvent extraction, likely due to transformation to reactive compounds that covalently bound to sediment organic matter.

TNT spiked to sediment degraded to aminated breakdown products during mixing, mostly 2ADNT (Table 5.b.4.A), with most breakdown occurring at the lowest treatment. Similarly, most of the spiked TNB was recovered as 1-amino-3, 5-dinitrobenzene (ADNB), also called 3,5-dinitroaniline, following mixing, with the degree of degradation decreasing with increasing concentration (Table 5.b.4.A). The recovery of spiked 2,4DANT was very low. No breakdown of 2ADNT was detected in spiked sediments.

For all compounds, concentrations measured at exposure termination were substantially lower than initial measured concentrations (Table 5.b.4.D), with a more pronounced decrease in concentrations occurring in TNB- and 2,4DANT-spiked sediments. For TNT-spiked sediments, the dominant compound in the 10-d sediment was 4ADNT, and for TNB-spiked sediments, the parent was not detected.

Mean survival was high in the control (78±10%) and tended to decrease with increasing concentrations for all nitroaromatic compounds (Fig. 5.b.4.A). Point estimates were calculated as 10-d LC50 values (Table 5.b.4.E) using sum molar concentration of all compounds as measured after spiking and mixing. LC50 values were very similar for the TNT, 2ADNT, and 2,4DANT experiments. The LC50 for the TNB experiment was approximately twice that calculated for TNT. For all nitroaromatic compounds, growth typically did not decrease or increase significantly in exposures to nitroaromatic compounds (Fig. 5.b.4.B); significant increasess in individual biomass were observed in one TNT and one TNB treatment where survival significantly decreased (Fig. 5.b.4.B).

Mixture toxicity experiments

For all experiments, expected concentrations approximated the targeted concentrations. As observed in the comparative toxicity experiments, measured sediment concentrations (Table 5.b.4.C) were lower than the expected concentrations. Similar to the comparative toxicity experiments, degradation of TNT and TNB was more pronounced in lower treatments. However, in the comparative toxicity experiments, formation of diaminotrinitrotoluenes occurred in 2ADNT-spiked sediments. As in single-compound experiments, measured concentrations of the mixture treatments were lower than the expected concentrations (Table 5.b.4.C). Because of degradation of spiked TNT and TNB, two compounds (4ADNT and ADNB), not present in the suite of compounds added to the sediment, were detected in the mixture sediments following mixing (Table 5.b.4.C).

In the single-compound experiments, mean mortality was low in the control (5%) and tended to increase with increasing concentrations for all nitroaromatic compounds (Fig. 5.b.4.A). Ten-d LC50 values were calculated using sum molar concentration of all compounds as measured after spiking and mixing (Table 5.b.4.E). In this set of experiments, the most acutely toxic compound was 2ADNT, followed by TNT, TNB and 2,4DANT (Table 5.b.4.E). Compared to values calculated in the comparative toxicity experiments, the LC50 values for TNT were very similar in both experiments, whereas the LC50 values for 2ADNT and TNB were lower in the mixture experiment, and the LC50 value for 2,4DANT was higher in the mixture experiment. For all nitroaromatic compounds, significant increases in individual biomass were observed in most treatments where survival was significantly decreased (Fig. 5.b.4.B).

Nominal concentrations of nitroaromatic compounds for each mixture treatment were targeted to yield equal nominal toxic units (or equal potency) for each compound (Table 5.b.4.F) and were determined using LC50 values derived the comparative toxicity experiments. For each compound in the mixture, measured toxic units were calculated using measured initial concentrations in the sediment and single-compound LC50s derived from exposures conducted concomitantly with the mixture exposures (Table 5.b.4.F). Because single-compound experiments were not performed for 4ADNT and ADNB (which appeared in the sediment due to the breakdown of TNT and TNB, respectively), the TU for 4ADNT and ADNB were determined using the LC50 value calculated for 2ADNT and TNB, respectively. Overall, measured STUs were higher than nominal STUs. Overall, ADNTs TUs contributed the most to the STU in the mixture treatments, followed by DANTs and ADNB (Table 5.b.4.F). Survival decreased with increasing STU (Fig. 5.b.4.C), with significant mortality occurring at STU of 0.78 and higher (Table 5.b.4.F). The sum toxic units for each treatment and the survival data were used in the calculation of a sum toxic unit LC50, which is expected to equal 1 when the compounds in the mixture interact in an additive manner, i.e., without decreasing (antagonism) or enhancing (synergism) the response of one another. The sum toxic unit LC50 was 1.15 (95% CI = 0.97-1.37) for the mixture. Because the LC50 value was only slightly higher then 1 and the 95% CI encompassed 1, response additivity likely occurs in sediment mixtures of TNT and its daughter compounds.

DISCUSSION

Derivation of accurate dose-response relationships using measured concentrations of TNT and its degradation products is challenging because of the rapid decrease in the concentration of solvent-extractable compounds. Because of rapidly changing exposure concentrations, the actual sediment concentration during the period when most mortality occurred was not determined in this study. Toxicity comparisons using concentrations measured at experiment set up, as provided in this study, assumes that the relative concentration decrease during the exposure period is similar to all compounds. Toxicity, summarized by 10-d LC50 values, was similar for a given compound between the two experiments and among all four compounds in a given experiment (Table 5.b.4.C), with differences never exceeding a factor of 3.

The sediment toxicity of TNT, TNB, and 2,4DANT to the midge *C. tentans* was has been previously studied (Steevens *et al.* 2002, see appendix A) via spiking to sediment collected from the same site as that used in this study. In 10-d exposures to spiked sediments, TNT was more toxic than the other two compounds used in that study, similar to the relative toxicity observed in this study. Although LC50 values were only reported for TNT because mortality in the high treatments did not bracket the range necessary for the calculation of that endpoint, comparison of dose response relationships suggest that mortality occurred at lower nominal concentrations in the present study compared to the study by Steevens *et al.* (2002).

The toxicity of TNT, TNB and 2,4DANT has also been investigated in sediment exposures using marine and estuarine invertebrates (Green et al, 1999; Lotufo *et al.* 2001, see Appendix A). Although the TNT exposure was investigated in an earlier study conducted in our laboratory, toxicity comparisons among the three compounds are facilitated by the use of sediment from the same site, similar spiking procedures and similar species and testing protocol. When nominal concentrations are used for sensitivity comparison for chronic effects on survival, the amphipod *Leptocheirus plumulosus* was substantially more sensitive to TNB than the polychaete *Neanthes arenaceodentata* but both species were similarly sensitive to TNT and 2,4DANT.

Interacting chemicals result in toxicological effects or responses that are difficult to predict based upon single chemical toxicological data. These interactions are defined as additive, synergistic, or antagonistic. Results from this investigation suggest that TNT and its daughter compounds interact datively when in a mixture in sediment exposures. Therefore, the toxicity of a mixture to a given receptor can be reasonably predicted using single-compound toxicity derived for that receptor. Moreover, comparative toxicity data suggest that TNT and its major breakdown products are similarly toxic to benthic invertebrates. Therefore, within this group of compounds, toxicity metrics determined for one compound can be used to predict the effect of another with an uncertainty factor of approximately 3-fold.

5.b.5. UNPUBLISHED STUDY 5: Toxicity of a heavy metal, PAH and nitroaromatic compound mixture. Gui Lotufo.

INTRODUCTION

Chemical contaminants in soil, sediment, or water are often present as a chemical mixture. However, the risks associated with contaminants are often considered on an individual chemical basis without consideration of chemical interactions affecting bioaccumulation and toxicity. Interacting chemicals result in toxicological effects or responses that are difficult to predict based upon single chemical toxicological data. These interactions are defined as additive (summation of toxic responses from multiple chemicals in proportion to the concentration of each chemical in the mixture; each chemical contributes to the resulting response or effect without modifying the mechanism or effect of other chemicals in the mixture), synergistic (toxic response is greater than would be predicted by additivity; chemicals enhance the toxicological effect of other chemicals) or antagonistic (toxic response is less than would be predicted by additivity; chemicals decrease the toxicological effect of other chemicals).

There is limited information available regarding the interaction of ordinance and energetic (OE) compounds with other contaminants such as metals, PCBs, PAHs, and pesticides. Current assumptions incorporate a significant degree of uncertainty in the risk assessment process. This uncertainty is mainly due to a lack of sound-scientific information. The proposed basic research functions as a starting point by which military relevant environmental mixtures can be more accurately assessed. By making more accurate assessments we decrease the chance of overestimating or underestimating the effects of a chemical mixture. An overestimation of the chemical hazards and risks will result in needless expense to the military for cleanup or remediation of military compounds. Conversely, an underestimation of the risks of military chemicals may result in significant and potentially irreversible environmental damage.

This investigation focuses on the interaction of an OE compound in a mixture with chemicals belonging to different classes of contaminants (*i.e.*, a PAH and a heavy metal).

MATERIALS AND METHODS

Experimental organisms

The amphipod *Hyalella azteca* were cultured at the Engineering Research and Development Center, Vicksburg, MS with organisms originally obtained from the USGS Biological Resources Division, Environmental and Contaminants Research Center (Columbia, MO). Species identity has been verified by a genetic differentiation study. Organisms were cultured in flow-through aged dechlorinated tap water and fed flake food (aquatic ecosystems) and hard maple tree leaves. Juvenile test organisms were collected by methods described by sieving with stacked sieves, a 600 µm on a 425 µm, and retaining organisms in the 425 µm sieve. Juvenile organisms collected were estimated to be 10 to 12 days of age based on a length of 2.12 ± 0.2 mm. Test organisms were acclimated to experimental conditions for 24 hours prior to initiation of all experiments.

Chemicals

The following compounds were used in the experiments: 1,3,5-trinitrobenzene (TNB) obtained from Supelco (Bellefonte, PA) (TNB), phenanthrene (PHE) and lead chloride ($PbCl_2$) obtained from Aldrich (Milwaukee, WI). Purity was >98% for all compounds.

Aqueous toxicity experiments

The mixture toxicity of TNB, PHE and lead was examined in 10-d exposures to aqueous solutions. In addition, single compound toxicity was determined in concomitant 10-d exposures. Five concentrations, selected using results from range-finding experiments, were used for each single compound (Table 5.b.5.A). Mixture treatments consisted of five equitoxic mixtures of TNB, PHE and lead targeting sum toxic units (STU) for the mixture ranging between 0.25 and 3 (Table 5.b.5.B). Each treatment corresponded to a target sum STU where each of 3 compounds was expected to contribute with one-third of the sum. Concentrations of single compounds in the mixture treatment were one-third the product of the target STU and the compound LC50. As an example, the treatment targeting a sum TU of 1 was prepared by adding each compound at a target concentration corresponding to one-third its LC50. Nominal 10-d LC50 values derived from preliminary experiments (Table 5.b.5.C) were used for calculation of target concentrations in the mixture treatments.

TNB and phenanthrene were added to exposure solutions dissolved in acetone. The concentration of acetone in all contaminant treatments and in the solvent control did not exceed 1.6 ml/L. A solvent control treatment was prepared using acetone only. Lead chloride solutions were prepared by adding aliquots of a concentrated stock (5 mg/ml) to dechlorinated water. Non-spiked dechlorinated water was used as a control for the lead experiment.

Exposure solutions were added to experimental chambers (300 ml tall beakers). Four replicates were used for each treatment. Ten juvenile amphipods were counted into 50 ml beakers and added to the exposure beakers. The exposure duration was 10 days. Two-thirds of the exposure water was exchanged every day. Animals were fed 0.5 ml of YCT (yeast-cerophyl-trout-chow) daily. Beakers were maintained in a water bath at 23°C under a 16h:8h light:dark cycle, with no aeration. Water quality parameters (dissolved oxygen, pH, hardness, conductivity, ammonia) were measured throughout the exposure. At termination of the exposure period, all surviving amphipods were retrieved from beakers and enumerated.

Calculations and statistical analysis

Completely randomized one-way analysis of variance was used to compare survival and growth data. William's test was used for comparing treatment means with control means. Significance level (a) was set at 0.05. Survival data were transformed by arcsine-square-root before analysis. Median lethal concentration (LC50) values were calculated using the trimmed Spearman-Karber method. For each mixture treatment, the toxic unit for each compound in the mixture was calculated by dividing its concentration by the LC50 derived from the single-compound exposure conducted concomitantly with the mixture experiment. For each mixture treatment, total toxic units (STU) were calculated as the sum of the individual compounds toxic units. Ten-d LC50 values, expressed as TU, were calculated using the STU from each treatment and survival data from individual replicates.

RESULTS

Aqueous Concentrations

Aqueous concentrations of TNB, phenanthrene and lead were measured in the stock aqueous solutions at experiment initiation and in the water collected from exposure beakers at day 1 before water renewal. For single compound exposures, measured concentrations were similar to nominal concentrations for phenanthrene and lead but were approximately half the nominal values for TNB (Table 5.b.5.A). Concentrations were also measured in the water collected from exposure beakers at day 1 before water renewal. Considerable loss occurred between water renewals (Table 5.b.5.A); the highest losses were observed for phenanthrene.

Concentrations determined for aqueous solution stocks in the mixture exposure were similar to nominal concentrations (Table 5.b.5.B). Compound loss over 24 h was highest for TNB and lowest for lead.

Single-compound toxicity

In the definitive single-compound experiments, no mortality occurred in control beakers (Fig. 5.b.5.A). Survival tended to decline with increasing concentrations for all compounds (Fig. 5.b.5.A). Ten-d LC50 values calculated for each compound (Table 5.b.5.C) indicate that lead was the most potent compound and TNB was the least potent compound.

Mixture Toxicity

Nominal concentrations of nitroaromatic compounds for each mixture treatment were selected to yield equal toxic units for each compound (Table 5.b.5.D) based on toxicity data from single-compound exposures. For each mixture treatment, actual toxic units were calculated for each compound using measured mean aqueous concentrations in the mixture and single-compound LC50s derived using measured water concentrations. Overall, expected TUs were similar to nominal TUs for lead. However, actual toxic units were higher than nominal toxic units for phenanthrene and TNB. Higher than expected TUs resulted from lower LC50 values for the mixture experiments compared to those from the previous experiments (Table 5.b.5.C). As a result of higher TUs for most compounds, actual sum TUs were also higher than nominal sum TUs.

Mortality increased with increasing sum toxic units in mixture exposures (Fig. 5.b.5.B). No significant mortality occurred at sum TU as high 1.69 and significant mortality occurred at 3.31 sum TU and higher treatments. The sum toxic units for each treatment and the survival data were used in the calculation of a sum toxic unit LC50, which is expected to equal 1 when the compounds in the mixture interact in an additive manner, i.e., without decreasing (antagonism) or enhancing (synergism) the response of one another. The sum toxic unit LC50 was 3.6 (95% confidence interval = 3.1-4.1) for the mixture. Because the LC50 value was substantially higher then 1 and the 95% confidence interval did not encompass 1, antagonistic interactions for the survival endpoint likely occurred in aqueous mixtures of TNB, PHE and lead.

DISCUSSION

This study suggests that the compounds TNB, phenanthrene and lead interact non-additively to promote mortality when in a mixture. Although results from the present study clearly indicate less-than-additive (i.e., antagonistic) toxicological interaction, further investigation of the mixture interaction among these compound is warranted before definitive conclusions about their mode of interaction are made. Only toxicological studies using mammals or microorganisms have focused on the interactions between TNT and other chemicals. A study by Levine *et al*. (1990) focused on the toxicological interaction between TNT and RDX utilizing F344 rats. Results of the study demonstrated that RDX antagonized the lethal effects of TNT. In a different study by Donnelly *et al*. (1998), the mutagenicity of the PAH benzo(a)pyrene (BaP), pentachlorophenol, and TNT were investigated in three bacterial strains. In all three strains, TNT significantly antagonized the mutagenicity of BaP. Both studies, in rats and bacteria, observed antagonism of other chemicals with TNT. Results from this study support the finding of less-than-additive effects observed in the present study. Therefore, assumptions of additivity in a risk assessment may overestimate the toxic effects of a chemical mixture containing TNT.

5.b.6. UNPUBLISHED STUDY 6: Toxicity and bioaccumulation of nitroaromatics and cyclonitramines from Picatinny Lake (Picatinny Arsenal, New Jersey). Gui Lotufo.

INTRODUCTION

Picatinny Arsenal (PTA) is located in northern New Jersey and was established in 1880. PTA has been involved in the production of explosives, rocket and munitions propellants. Picatinny Lake has received numerous wastewater discharges, including effluent from the explosives manufacturing wastewater treatment plant. A large suite of explosives was detected at high concentrations in sediment samples from Site 40 in Picatinny Lake. Sediment from this location was used to investigate the toxicity and bioaccumulation of sediment-associated explosive compounds and their breakdown products in benthic invertebrates. Highly contaminated Site 40 sediment was diluted with relatively uncontaminated Lake Picatinny sediment to produce a dilution series. In addition, a toxicity experiment was conducted using decreasing dilution concentrations of the porewater extracted from the contaminated sediment. Results from these experiments were interpreted using single-compound sediment toxicity data generated from spiked-sediment exposures.

MATERIALS AND METHODS

Picatinny Lake Sediment Collection

Contaminated and reference sediment were collected in the spring of 2001 from Picatinny Lake. Sediments were collected from Site 40 location 40SD-3 by Science Applications International Corporation (SAIC) personnel. Sediment 40SD-3 was used as a source of contaminated sediment. Lake Picatinny sediment with very low concentrations of ordnance compounds and a similar granulometric profile as sediment from station 40SD-3 was also collected and used for serially diluting 40SD-3 sediment. This relatively uncontaminated sediment was also used as reference sediment for the toxicity and bioaccumulation experiments.

Control Sediment Collection

Sediment used as the performance control sediment was collected from Brown's Lake located at the Engineer Research and Development Center in Vicksburg, MS. Sediment was collected using a hand shovel (top 10 cm layer), placed in 5 gallon plastic buckets and stored in a cold room at 5-6^{o}C until use. Brown's Lake sediment was mainly silty material with 1.8% sand, 98.2% fines (clay and silt), and 0.65% total organic carbon. Concentrations of PAHs, heavy metals, and pesticides were either below detection levels or at concentrations not associated with adverse effects to aquatic invertebrates.

Sediment Dilution Series Preparation

A dilution series (100, 50, 25, 12, 6, 3%) of the 40SD-3 sediment was prepared by vigorously mixing the appropriate volume of 100% 40SD-3 sediment with a known volume of reference sediment for 8 hours. Dilution concentrations were prepared on a dry weight basis, though contaminated and reference sediments were mixed wet. Mixed sediments were held at 4 °C for 21 d. After this period, sediments were allowed to warm to room temperature and homogenized before use in the experiments. Sediments also were centrifuged at 5,000*g* for porewater extraction. Porewater samples were analyzed for nitroaromatic and cyclonitramines within 48 hours from extraction.

Porewater Dilution Series Preparation

Approximately 1 L of the 40SD-3 sediment was centrifuged at 5,000*g* for porewater extraction. A dilution series (8, 6, 4, 3, 2, and 1%) of the stock porewater was prepared by mixing an appropriate volume with dechlorinated water to create a final volume of 0.5 L.

Experimental Organisms

Lumbriculus variegatus was used in the bioaccumulation experiment and *H. azteca* in the toxicity experiment. Both organisms are cultured in the ERDC-EERT laboratory following standard operating procedures. Organisms are cultured in flow-through environmental chambers and fed 3 times weekly. Cultures were started using organisms originally obtained from the U.S.Geological Survey (Columbia, MO). *L. variegatus* are cultured in 20 L aquaria containing soaked brown paper towels as a substrate and food source. *H. azteca* are cultured in 20 L aquaria containing plastic webbed coil material for a substrate and fed flake food and soft maple leaves. Water quality parameters (temperature, DO, pH) were determined weekly, and toxicant reference tests were conducted monthly. A reference toxicant test was set-up concurrent to each test to evaluate the health of the test organisms and suitability of the test conditions.

Sediment Bioaccumulation Experiment

The U.S.EPA (2000) *Lumbriculus variegatus* 28-day bioaccumulation test for sediments (Test Method 100.3) was used, with modifications, to investigate bioaccumulation of contaminants from Picatinny Lake dilution series sediments. Five replicates of each sediment treatment (control, reference, 40SD-3, 50, 25, 12, 6, and 3% dilution) were used. Tests were conducted under static conditions in 300 ml glass beakers containing 100 g sediment and 150 ml of overlying water. At the initiation of the bioaccumulation test, 10 mature worms were added to each beaker. Animals were not fed during the experiment. Light aeration was provided to maintain dissolved oxygen concentrations. Temperature was maintained at 23± 1 °C and the light:dark photo cycle was 16:8 using white light. Water quality parameters (conductivity, hardness, pH, alkalinity, ammonia, temperature and dissolved oxygen) were measured at test initiation and termination. Temperature and DO were monitored daily. The intended exposure period was 28 days; however, high mortality in Picatinny Lake sediment treatments resulted in experiment termination after 4 days. At the end of the exposure period, test sediments were sieved to recover surviving worms, which were placed in glass culture bowls and enumerated.

Sediment Toxicity Experiment

The U.S.EPA (2000) *Hyalella azteca* 10-day survival and growth test (Test Method 100.1) was used to investigate the toxicity associated with Picatinny Lake dilution series sediments. Five replicates were used for each treatment, including the reference sediment and the control sediment. Toxicity tests were conducted utilizing a flow-through system. Water splitter chambers were placed over the 300ml glass beakers and 2L of test water (dechlorinated tap water) was delivered to the beakers every 12 hours. Each beaker contained 4.5 cm of homogenized sediment and 10 ten-day old amphipods. Animals were fed 1 ml (1.8 mg/ml) of YCT (mixture of yeast, cerophyll, and trout chow) daily. Initial and final water quality parameters for dissolved oxygen (DO), pH, temperature, ammonia, conductivity, hardness, and alkalinity were determined. Temperature and DO (not to fall below 2.5 mg/L) were monitored daily. At the end of the exposure, sediments were sieved to recover the organisms and document survival.

Diluted Porewater Toxicity Experiment

Solutions were added to experimental chambers (100 ml beakers). Three replicates were used for each treatment. Ten juvenile *H. azteca* were counted and added to the exposure beakers. The exposure duration was 4 days. Two-thirds of the exposure water was exchanged after 48 h. Beakers were maintained in a water bath at 23°C under a 16h:8h light:dark cycle. At termination of the exposure period, water quality parameters (dissolved oxygen, pH, hardness, conductivity, ammonia) were measured and all surviving amphipods were retrieved from beakers and enumerated. Surviving animals were blotted dry and weighed.

RESULTS

Chemical analysis of 40SD-3 sediment dilution series

Chemical analysis of Picatinny Lake sediment revealed that RDX, HMX, TNT, 2ADNT (2-aminodinitrotuene), 4ADNT (4-aminodinitrotoluene), 2,4DANT (2,4-diaminonitrotoluene) and TNB (trinintrobenzene) were present in 40SD-3 sediment, but not in the reference sediment

Concentrations of contaminants in diluted sediments are reported in Table 5.b.6.A. The compounds HMX, TNT, RDX and 4ADNT were detected in all treatments. The compound 2ADNT, 2,4DANT and TNB were not detected in the lowest treatment. Overall, the relative concentration of compounds in the sediment corresponded to the nominal dilution of contaminated sediment with contaminant-free sediment (Table 5.b.6.B).

Compound concentration in the porewater of 40SD-3 sediment (Table 5.b.6.C, 100% treatment) was close to the solubility limit for HMX, TNT and RDX. Dilution of the sediment with uncontaminated sediment did not result in a decrease in porewater concentrations of those compounds. Porewater concentrations remained relatively unchanged across dilution series treatments, on an absolute (Table 5.b.6.C) or relative basis (Table 5.b.6.D). Consequently, the concentrations of compounds in the porewater relative to the concentration of compound in the sediment increased with increasing dilution of the sediment for these compounds (Table 5.b.6.E).

Sediment toxicity

Survival in the control and reference sediment replicates was 60% and higher for *H. azteca* and 100% for *L. variegatus*. Mean survival in controls and the dilution series treatments are presented in Fig. 5.b.6.A. Survival was low in the 3% treatment of *L. variegatus*. Complete mortality occurred in all treatments for *H. azteca* and in the 6% and higher treatments for *L. variegatus* (Fig. 5.b.6.A).

Bioaccumulation

The bioaccumulation experiment was not completed successfully because most of the test-organisms died within the initial few days of exposure. Therefore, no tissue residue data are reported.

Chemical analysis of porewater dilution series

Concentrations of nitroaromatics and cyclonitramines in the stock and diluted sediment 40SD-3 porewater are presented in Table 5.b.6.A. In a manner different from the porewater obtained from diluted sediments, the compound concentration in the porewater dilution series decreased according to the dilution factor (Table 5.b.6.F).

Porewater dilution series toxicity

Amphipod survival was high in the control and significantly decreased in all diluted porewater treatments (Fig. 5.b.6.B). Complete mortality was observed in the 4% dilution and higher treatments.

DISCUSSION

The concentrations of explosive compounds in the sediment decreased with increasing dilution of the stock contaminated sediment. However, concentrations in the porewater of diluted sediments remained relatively unchanged. The porewater concentrations of TNT, RDX and HMX in 100% 40SD-3 were close to the solubility limit for these compounds (Table 5.b.6.C). Dilutions below 3% 40SD-3 sediment would be necessary to promote a decrease in porewater concentration.

Sediment toxicity to benthic amphipods can be relatively accurately interpreted by comparing porewater concentrations to toxic water-only concentrations. The compounds RDX and HMX were not toxic to *H. azteca* in water exposure up to the solubility limit of those compounds (Table 5.b.6.G). TNT was lethally toxic to *H. azteca* at approximately 2 mg/L, a concentration much lower than porewater concentrations for all dilution series treatments (Table 5.b.6.G).

A gradient of contaminant concentrations was successfully created by diluting full-strength 40SD-3 porewater with uncontaminated water. Complete mortality occurred at 4% and higher treatments, where TNT concentrations exceeded the 10-d LC50 determined for *H. azteca*. Four-day single LC50 values for all compounds detected in the 40SD-3 sediment porewater are being generated using *H. azteca*. These values will permit testing the predictive power of using the toxic units approach for mixtures to predict toxicity of field-contaminated samples.

The objective of our study was to obtain a range of biological responses to diluted Lake Picatinny sediments. This objective could not be met because the concentration of nitroaromatic compounds in the sediment and in the porewater of all dilution treatments far exceeded lethal concentrations for *H. azteca*. Sediment 40SD-3 would have to be diluted further to create suitable concentrations for use in *H. azteca* toxicity tests. We estimated that a dilution down to 0.1% would be necessary to generate sediments within the range necessary to promote partial survival of *H. azteca* in sediment exposures. Reasonable and consistent achievement of this degree of sediment dilution would be difficult if not impossible to reach.

SEDIMENT RESAMPLING EFFORTS

With the objective of obtaining sediments from Picatinny Lake considerably less contaminated than sediments collected in 2001 (Table 5.b.6.A), we arranged for sediment sampling in the Lake in 2002. Sediments collected from or around site 40SD-3 in September and in November 2002 were not suitable for use in our experiment because the concentrations of explosive compounds were too low (Table 5.b.6.G) to promote toxicity in benthic invertebrates previously used in our studies.

5.b.7. UNPUBLISHED STUDY 7: Toxicity and critical body burden of TNT in the freshwater midge *Chironomus tentans* in aqueous exposures. Gui Lotufo.

INTRODUCTION

The aqueous toxicity of TNT has been previously studied for a few species of aquatic invertebrates (Talmage *et al*. 1999). However, the bioaccumulation of TNT in aquatic species is poorly known. Therefore, further studies are necessary to enhance our understanding of TNT bioaccumulation in aquatic organisms and of the relationship between compound bioaccumulation and ecologically relevant toxicity endpoints, such as survival. The objective of this study was to derive critical body residues for a benthic invertebrate through the relationship between survival and body residue expressed either as total bioaccumulated compounds, determined using radioactivity, and as the sum molar concentration of TNT and its degradation products that are extractable and quantifiable via chemical analysis.

MATERIALS AND METHODS

Experimental organisms

Chironomus tentans were obtained as egg masses from Environmental Consulting and Testing (Superior, WI). Upon arrival at the laboratory, egg masses were placed in plastic tote tubs containing dechorinated water and a thin layer of sand. Fish food flakes (Tetrafin®, Tetra Sales) were added daily. Larvae were reared to the forth instar based on a head capsule width (USEPA, 2000).

Exposure media

A TNT spiking stock was prepared for each water treatment by combining ^{14}C-labeled TNT to non-radiolabeled TNT in the appropriate volume of acetone. Radiolabeled trinitrotoluene (^{14}C-TNT, 23.6 Ci/mol, 99% radiochemical purity) was purchased from New England Nuclear Research Products (Boston, MA). Non-radiolabeled TNT (99% purity) was purchased from Chem Service (Westchester, PA). Exposure water was prepared by spiking 0.5 ml of TNT acetone stock to each liter of artificial seawater. The target radioactivity in all exposure water treatments was 5,000 dpm/ml. The target TNT concentration in water treatments ranged from 0.25 to 6 mg/L (Table 1). The control treatment consisted of a 0.5 ml/L acetone solution. The specific activity of the isotopically diluted ^{14}C-TNT in each spiking solution, determined by analyzing freshly spiked water for radioactivity and TNT concentration (see below), ranged from 0.089 to 2.189 Ci/mol.

Aqueous toxicity experiments

Toxicity and bioaccumulation of ^{14}C-TNT were examined in 4-d water-only exposures. Midges were exposed to a range of contaminant concentrations (Table 5.b.7.A) in 300 ml tall beakers filled with 250 ml of test solution and 5 g of quartz sand as tube-building substrate. Four beakers were used for each treatment. Each exposure beaker received 10 organisms at experiment initiation. Beakers were placed in a water bath at 23°C and received trickle flow aeration under gold fluorescent lights at a 16h:8h light:dark cycle and food was provided every day as 6 mg of ground TetraFin per beaker. Each day during the duration of the experiment, the number of larvae in each beaker was recorded and dead animals were removed. Approximately 90% of the exposure solution from each beaker was renewed every 24 h. Exposure water was sampled for radioactivity at the beginning of the experiment and daily thereafter before and after each renewal. This procedure was conducted for monitoring compound concentration throughout the exposure period and to determine compound volatilization and degradation following the 24-h period preceding each exposure solution exchange event. One milliliter of water was transferred to 12-ml scintillation cocktail (3a70b; Research Products International, IL) and ^{14}C-activity was quantified by liquid scintillation counting (LSC) on a Tricarb Liquid Scintillation Analyzer (Model 2500; Packard Instruments, Meridien, CT). For water chemical analysis, samples were taken at experiment initiation and at day 1 before water renewal. For tissue radioactivity and chemical concentration determinations, midges were removed from beakers at experiment termination. For each beaker, three larvae were blotted dry, weighed, and transferred to 12-ml of 3a70b scintillation cocktail, and analyzed for radioactivity as described above. Remaining animals from each beaker were rinsed with water, blotted dry, weighed, and analyzed for nitroaromatic compounds in tissues (see below). Measured specific activities were used for converting the radioactivity concentration in water or tissue samples to TNT molar-equivalent concentration, and therefore includes parent compound and breakdown or conjugated compounds.

Chemical analysis

Water samples. Aqueous samples were analyzed for TNT and its daughter compounds animodinitrotuenes (2ADNT and 4ADNT) and diaminonitrotoluenes (2,4DANT and 2,6DANTs) using a modified version of U.S.EPA method 8330. Chemical analysis for TNT and degradation products in water was conducted using an Agilent 1100 HPLC (Palo Alto, CA, USA) equipped with a diode-array detector (USEPA 1997). The column used was a SUPELCO RP-Amide C-16 with a sample injection volume of 100 µL and flow rate of 1 ml/min. Solvent ratios were 45% water and 55% methanol and UV absorbance was measured at 230 nm. Laboratory reporting limits for the analysis of water samples using this method are 0.1 ppm for TNT, TNB, 2ADNT, 4ADNT, and 2,4DANT.

Tissue samples. Tissue samples ranging from 6 to 47 mg wet wt. (28 ± 11 mg) were placed in bead-beating vials with 50 mg of 0.5-mm glass beads and 0.75 ml of HPLC grade acetonitrile. Samples were homogenized on a mini beadbeater (Biospec, Barttlesville, OK) for 100 sec at 4200 oscillations/min and placed on ice. Samples received 0.75 ml of 1% $CaCl_2$ and were sonicated (Branson 3200, Branson Ultrasonics, Danbury, CT) for 1 h at 18°C in a water bath (Neslab RTE-111, Neslab Instruments, Newington, NY). Samples were centrifuged for 10 min at 7500 x g at 4°C. The supernatants were filtered through 0.45 µm PTFE syringe filters (Nalge Nunc, Rochester, NY) into amber sample vials. Analytes were isolated and identified as described for water samples.

Statistical analysis and bioaccumulation modeling

One-way analysis of variance was used to analyze amphipod survival data. Contaminant treatments were compared to the control treatment using the William's test. Significance level (a) was set at 0.05. Survival data were transformed by arcsine-square-root before analysis. Median lethal concentration (LC50) values were calculated using the trimmed Spearman-Karber method.

RESULTS

Aqueous concentrations

The concentration of TNT-molar equivalents in the exposure solutions, measured using LSC, were similar to the target concentrations and remained relatively constant during the exposure period, as little decrease in radioactivity occurred following the 24-h period between exposure solution exchanges (Table 5.b.7.A). However, HPLC-measured concentrations in the exposure solutions were lower relative to the LSC-measured concentrations. Most of the TNT in the freshly spiked solution degraded to 4ADNT and the sum concentration of TNT and 4ADNT decreased substantially following the 24-h period between exposure solution exchanges (Table 5.b.7.A).

Bioaccumulation

TNT and TNT aminated breakdown products were detected in solvent extracts of larval midges following a 4-d exposure to TNT. Body residues were estimated as TNT molar equivalents using radioactivity and as sum molar concentration of TNT, ADNTs and DANTs using solvent extraction and HPLC quantification (Table 5.b.7.B). The HPLC-measured body residues were only a small fraction (3-14%) of the LSC-determined tissue concentrations. Body residue increased with increasing water concentration. Overall, the ADNTs were the dominant compound in tissue solvent extracts, with the relative fraction of breakdown products decreasing with increasing water concentration. Bioconcentration factors were substantially higher when calculated using LSC-measured tissue and water concentrations than when concentrations were measured via HPLC. BCF values were substantially more variable across treatments when calculated using HPLC-determined concentrations (Table 5.b.7.B).

Aqueous toxicity and critical body residues

At termination of the 4-d exposure, survival was high in the control treatment (94±9%) and treatments 1.1 to 8.8 µmol/L (83-87%), but was significantly decreased in the two highest treatments (Table 5.b.7.B). The 4-d LC50 was 8.54 µmo/L (Table 5.b.7.C).

The relationship between body residue and survival is presented in Fig. 5.b.7.B. Only the body residues measured in the midges from the 17.6 µmol/L treatment were associated with significant decrease in survival. The body residue in dead organisms from the highest treatment was not determined. The median lethal residue for LSC-measured body residues was substantially higher than the value for HPLC-measure residues (Table 5.b.7.C).

DISCUSSION

Breakdown of TNT and loss of TNT and TNT breakdown products over time in aqueous solutions were likely due to the addition of food (ground fish flakes), as lesser degradation and losses were observed in similar experiments where food was not added (Lotufo, unpublished).

The potential for TNT to bioconcentrate in aquatic species is expected to be low given the low hydrophobicity of this chemical (measured and calculated log K_{ow} values of 1.6-2.7). The only bioconcentration factors for aquatic animals reported in the literature (Liu *et al.* 1983) were derived using ^{14}C-activity as a surrogate for TNT bioaccumulation. The range of values (202 – 453) for invertebrates and fish was higher than the range of values observed for *C. tentans* in this study (85 – 129). Values reported in Liu *et al.* (1983) and in this study using ^{14}C-activity were substantially higher than the expected value of 20 calculated using a log K_{ow} of 2.03 for TNT. Values derived in this study using HPLC-measured water and tissue sum molar concentrations of TNT and its daughter compounds (5.3 – 20.5) were in agreement with the predicted value. The reason for the difference between ^{14}C-activity and HPLC derived BCF values is the solvent-resistance of a sizable fraction (20-30%) of radiolabeled ^{14}C measured in the tissue and the portion of the extractable activity that corresponded to highly polar TNT metabolites that are not quantifiable using existing standards and analytical procedures (Lotufo and Steevens, unpublished). Covalent binding of metabolic products to organic molecules likely renders them resistant to solvent extraction, as has been observed for TNT and human hemoglobin (Bakhtiar and Leung 1997).

We successfully derived critical body residues (CBRs), here expressed as median lethal residues, for the sum of TNT and its major breakdown products and also for all the radiolabeled molecules. The CBRs for HPLC-identified compounds was substantially higher than that calculated for all the bioaccumulated molecules using radioactivity. The toxicological significance of non-extractable and non-detected compounds is still unknown and further investigation is warranted.

5.b.8. UNPUBLISHED STUDY 8: Toxicity and critical body burden of TNT in the amphipod *Leptocheirus plumulosus* in aqueous exposures. Gui Lotufo.

INTRODUCTION

The aqueous toxicity of TNT has never been investigated using estuarine or marine aquatic invertebrates (Talmage *et al*. 1999). Moreover, the bioaccumulation of TNT in aquatic species is poorly known. The only bioconcentration factors reported in the literature (Liu *et al*. 1983) were derived using ^{14}C-activity as a surrogate for TNT bioaccumulation. Therefore, further studies are necessary to enhance our understanding of TNT bioaccumulation in aquatic organisms and of the relationship between compound bioaccumulation and ecologically relevant toxicity endpoints, such as survival. Accurate assessment of the relationship between environmental concentrations and the manifestation of toxicity is only possible when toxicokinetic parameters are measured (Lotufo *et al.*, 2000). The objective of this study was to investigate the toxicity and the bioaccumulation kinetics (rates of uptake and elimination) of TNT for the estuarine amphipod *Leptocheirus plumulosus*. In addition, we examined the relationship between survival and body residue to derive critical body residues for this species.

MATERIALS AND METHODS

Experimental organisms

Leptocheirus plumulosus were laboratory cultured as previously described (Emery *et al*. 1997). Cultures were maintained in 42 x 24 x 15 cm polyethylene tote boxes containing 2 to 3 cm of sediment (Sequim Bay, WA) and 3 L of artificial seawater (40 Fathoms®, Marine Enterprise International, Baltimore, MD) at a salinity of 20. Cultures were held under a 16:8 light:dark cycle, at 23 ?C and with trickle flow aeration. Animals were fed ground TetraMin® (Tetra Sales, Blacksburg, VA) and sixty percent of the overlying water was renewed three times weekly.

Exposure media

A TNT spiking stock was prepared for each water treatment by combining ^{14}C-labeled TNT to non-radiolabeled TNT in the appropriate volume of acetone. Radiolabeled trinitrotoluene (23.6 Ci/mol, 99% radiochemical purity) was purchased from New England Nuclear Research Products (Boston, MA). Non-radiolabeled TNT (99% purity) was purchased from Chem Service (Westchester, PA). Exposure water was prepared by spiking 0.5 ml of TNT acetone stock to each liter of artificial seawater. The target radioactivity in all exposure water treatments was 5,000 dpm/ml. The target TNT concentration in water treatments ranged from 0.25 to 6 mg/L (Table 5.b.8.A). The control treatment consisted of a 0.5 ml/L acetone solution. The specific activity of the isotopically diluted [^{14}C]TNT in each spiking solution, determined by analyzing freshly spiked water for radioactivity and TNT concentration (see below), ranged from 0.096 to 2.098 Ci/mol.

Aqueous toxicity experiments

Toxicity and bioaccumulation of ^{14}C-TNT were examined in 8-d water-only exposures. Amphipods were exposed to a range of contaminant concentrations (Table 5.b.8.A) in 600 ml beakers filled with 500 ml of test solution. Nine beakers were used for each treatment. Each exposure beaker received 10 organisms at experiment initiation. Beakers were placed in a water bath at 23°C and received trickle flow aeration under gold fluorescent lights at a 16:h-8h light:dark cycle and food was provided every day as 10 mg of ground Tetramin per beaker. Each day of the experiment, the number of amphipods in each beaker was recorded and dead amphipods were removed. Approximately 50% of the exposure solution from each beaker was renewed every 24 h. Exposure water was sampled for radioactivity and chemical analysis at the beginning of the experiment and daily thereafter before and after each exposure water renewal for monitoring compound concentration throughout the exposure period and to determined compound volatilization and degradation following the 24-h period preceding each exposure solution exchange event. One milliliter of water was transferred to 12-ml scintillation cocktail (3a70b; Research Products International, IL) and ^{14}C-activity was quantified by liquid scintillation counting (LSC) on a Tricarb Liquid Scintillation Analyzer (Model 2500; Packard Instruments, Meridien, CT). For tissue radioactivity and chemical concentration determinations, all amphipods were removed from two beakers at days 1 and 4 and from five beakers at day 8. For each beaker, three amphipods were blotted dry, weighed, and transferred to 12-ml scintillation cocktail, sonicated for 20 sec with a Branson Sonifier 450 high intensity probe-sonicator (Danbury, CT) at 50% power, and analyzed for radioactivity as described above. Measured specific activities were used for converting the radioactivity concentration in water or amphipod samples to TNT molar-equivalent concentration, and therefore includes parent compound and breakdown or conjugated compounds. Remaining amphipods from each beaker were rinsed with water, blotted dry, weighed, and frozen at –80°C for chemical analysis of nitroaromatic compounds in tissues.

Chemical analysis

Water samples. Aqueous samples were analyzed for TNT and its daughter compounds animodinitrotuenes (2ADNT and 4ADNT) and diaminonitrotoluenes (2,4DANT and 2,6DANTs) using a modified version of U.S.EPA method 8330. Chemical analysis for TNT and degradation products in water was conducted using an Agilent 1100 HPLC (Palo Alto, CA, USA) equipped with a diode-array detector (USEPA 1997). The column used was a SUPELCO RP-Amide C-16 with a sample injection volume of 100 µL and flow rate of 1 ml/min. Solvent ratios were 45% water and 55% methanol and UV absorbance was measured at 230 nm. Laboratory reporting limits for the analysis of water samples using this method are 0.1 ppm for TNT, TNB, 2ADNT, 4ADNT, and 2,4DANT.

Tissue samples. Tissue samples ranging from 6 to 47 mg wet wt. (28 ± 11 mg) were placed in bead-beating vials with 50 mg of 0.5-mm glass beads and 0.75 ml of HPLC grade acetonitrile. Samples were homogenized on a mini beadbeater (Biospec, Barttlesville, OK) for 100 sec at 4200 oscillations/min and placed on ice. Samples received 0.75 ml of 1% $CaCl_2$ and were sonicated (Branson 3200, Branson Ultrasonics, Danbury, CT) for 1 h at 18°C in a water bath (Neslab RTE-111, Neslab Instruments, Newington, NY). Samples were centrifuged for 10 min at 7500 x g at 4°C. The supernatants were filtered through 0.45 µm PTFE syringe filters (Nalge Nunc, Rochester, NY) into amber sample vials. Analytes were isolated and identified as described for water samples.

Statistical analysis and bioaccumulation modeling

All measurement values are expressed as a mean ± 1 standard deviation. Completely randomized one-way analysis of variance was used to compare survival data. William's test was used for comparing treatment means with control means. Significance level (a) was set at 0.05. Survival data were transformed by arcsine-square-root before analysis. Median lethal concentration (LC50) and median lethal tissue residue (LR50) values were calculated using the trimmed Spearman-Karber method.

Accumulation data from the 8-d experiment were fit to a two-compartment model (Landrum *et al.*, 1992):

$C_a = k_s * C_w * k_e (1 - e^{-ket})$,

where C_a is the concentration in the animal (µmol [g wet wt]$^{-1}$), k_s is the conditional uptake clearance rate coefficient (g dry sediment [g wet tissue]$^{-1}$ h^{-1}), C_w is the concentration in the water (µmol L^{-1}), k_e is the conditional elimination rate constant (h^{-1}), and t is time (h). The corresponding half-lives ($t_{1/2}$) were determined in terms of k_d by the formula ($t_{1/2}$) = 0.693 k_d^{-1}. Analyses were performed using Sigma Plot® (Release 6.0, SPSS, Chicago, IL).

RESULTS

Aqueous concentrations

The concentration of TNT-molar equivalents in the exposure solutions, measured using LSC, were similar to the target concentrations and remained relatively constant during the exposure period, as little decrease in radioactivity occurred following the 24-h period between exposure solution exchanges (Table 5.b.8.A). Concentrations measured using HPLC were substantially lower than LSC-measured concentrations for the low treatments, but were increasingly similar to the latter with increasing TNT loading in the water (Table 5.b.8.A). Substantial degradation of TNT to 4ADNT occurred in the low treatments, but virtually no degradation occurred in the high treatments. Whereas very little loss of compound was measured using LSC, the HPLC-measured sum concentration of TNT and 4ADNT decreased substantially following the 24-h period between exposure-solution exchanges for the low treatments, but remained relatively unchanged in the high treatments (Table 5.b.8.A).

Bioaccumulation

Uptake data was collected following 24, 96, and 192 h of exposure to radiolabeled TNT. Kinetics data were not collected for the two highest treatments because of complete mortality before the 96 h sampling period. Concentrations in amphipod solvent extracts were below detection limit for all treatments at all sampling periods. Therefore, toxicokinetics data were generated using only LSC-measured tissue concentrations. The uptake rate constants varied by a factor of 4 across treatments. The model-derived elimination rate was not significantly different from zero for the highest treatment. The elimination rate constants varied by a factor of 3 across treatments (highest excluded). No apparent relationship was evident between exposure concentration and uptake or elimination rates. Kinetically-derived (K_u / K_e) and day-8 measured BCF values were very similar, as steady-state was approaching at the 8-d exposure period.

Aqueous toxicity and lethal body residue

Mean survival remained high in the control treatment (> 88%) and treatments 1.1 to 4.4 µmol/L (> 76%) throughout the 8-d exposure (Fig. 5.b.8.B). All amphipods in the 17.6 and 26.4 µmol/L treatments died between days 2 and 4 of the experiment. Survival was significantly decreased in treatment 8.8 µmol/L between days 4 and 8 of the exposure. The 4-d LC50 value was approximately twice as high as the 8-d LC50 (Table 5.b.8.C). Survival and body residue data at days 4 and 8 were used for the calculation of median lethal residues. The 4-d LR50 value was very similar to the 8-d LR50 (Table 5.b.8.C).

DISCUSSION

TNT and TNT major metabolites were below detection level in amphipod tissue solvent extracts using HPLC analysis. Therefore, body burdens were only reported as TNT molar equivalents estimated using ^{14}C-activity in the whole body. The kinetics parameters derived in the present study revealed that the elimination of tissue-associated ^{14}C-radiolabel resulting from exposure to TNT is slow (elimination half-live ~ 30-70 h). These slow elimination rates were unexpectedly slow relative to the hydrophobicity of TNT (log K_{ow} ~ 2), as rates for TNT were similar to those measured for DDT (log K_{ow} ~ 6), a much more hydrophobic compound (Lotufo, unpublished). The reason for the low elimination rate of radiolabeled compound is likely due to the binding of TNT and its metabolites to organic molecules within the organism, as demonstrated in previous studies from our laboratory (Lotufo and Steevens, unpublished). Covalent binding of TNT and its metabolic products to organic molecules renders these molecules resistant to solvent extraction and likely removes them from the pool of molecules that can cross biological membranes and hence leave the organism.

Critical body residues (CBRs) calculated as median lethal residues for TNT molar equivalents estimated using ^{14}C-activity in the tissues were very similar to those calculated for C. tentans in the previous study.

5.b.9. UNPUBLISHED STUDY 9: Correlation between histone 1a gene expression and contaminant exposure in *Chironomus tentans.* Ed Perkins.

Introduction

TNT is a common explosive chemical used in the manufacturing of ordnances. Past waste disposal practices at Army ammunition plants and ongoing military training activities have resulted in the contamination of soil, sediment and water with TNT. Exposure to contaminated media is a potential health risk. Several studies in terrestrial and aquatic systems have demonstrated toxic effects at the organism level (Talmage 1999, ATSDR 2002). However, mechanisms through which TNT may initiate damage at the cellular is largely unknown (ATSDR 2002).

Most evidence for the mechanism of toxicity of TNT is derived from acute exposure mammalian studies. Metabolism most likely occurs in the liver through both reductive and oxidative processes (Leung, 1995). TNT is rapidly metabolized to hydroxylamines that subsequently bind to cell components, is conjugated, or eliminated. The metabolism of TNT may also produce reactive oxygen radicals, damaging DNA, proteins, and lipids (Homma-Takeda *et al*., 2002; Bakhtiar *et al*., 1997, Liu *et al*., 1992). Observations of enhanced growth at low levels and reduced growth and survival at high levels of TNT in invertebrates (*Chironomus tentans*) suggests complex interactions between cellular function and gene expression (Steevens *et al*., 2002).

Effects of TNT on growth may result from different toxicological effects at the cellular level. TNT may directly impact cell growth by modifying nuclear proteins involved in chromosomal structure and gene regulation. Histone proteins are essential nuclear proteins that form the scaffolding upon which chromosomal DNA is condensed and are highly conserved across most organisms. The histone proteins H2a, b and H3a, b form the core around which DNA is wound. Linker histones H1a –e are involved in regulation of gene specific expression (Georgel and Hansen 2001, Khochbin 2001). Expression of h1a is required for proper regulation of pyruvate decarboxylase gene expression in *Neurospora crassa* (Folco *et al*., 2003). Histone proteins are intimately tied to nuclear organization and expression of histone mRNA which has been linked to growth and the rate of DNA synthesis (Plumb *et al*., 1984, Wang *et al*. 1997). Since expression of h1a is likely to be affected by any chemical that impacts growth and survival, toxic levels of other priority pollutants such as pesticides, polyaromatic hydrocarbons, and metals may also affect h1a.

We have examined linker histone h1a expression in *C. tentans* and linked expression levels to growth and survival to better understand the impact of TNT exposure. A semiquantitative reverse transcriptase polymerase chain reaction assay was developed for the histone 1a gene in *C. tentans*. We also examined whether any impacts seen were specific to TNT or if other priority pollutants such as DDT, phenanthrene, fluoranthene, cadmium, copper, and zinc also affected h1a expression. Expression of h1a responded in a dose dependent manner when exposed to TNT and other chemicals and was proportional to effects measured at the whole organism level.

Materials and Methods
Experimental organisms

Chironomus tentans were obtained as egg masses from Environmental Consulting and Testing (Superior, WI). Upon arrival at the laboratory, egg masses were placed in plastic tote tubs containing dechlorinated water and a thin layer of sand. Fish food flakes (Tetrafin®, Tetra Sales) were added daily. Larvae were reared for 10 or 11 days after hatching to the third instar based on a head capsule width ranging from 0.33 to 0.45 mm (USEPA, 2000).

Animal exposures

Chironomus larvae were exposed to different levels of TNT over 10 days. Triplicate exposures were conducted with nominal concentrations of 0.1, 0.5, 1.0 and 2.0 mg/LTNT. TNT (99% purity) was purchased from Chem Service (Westchester, PA, USA). Replicate exposures were conducted for 1 hr, 24 hr, 96 hr, and 240 hrs at each concentration. A triplicate, unexposed control was exposed for 240 hrs. TNT dissolved in acetone was added to dechlorinated tap water (1.6 ml acetone /L) for preparing aqueous exposure solutions. Exposure chambers were 300-ml beakers filled with 250 ml of exposure solution. Each beaker received 10 larval midges. Three replicates were used for each treatment. Beakers were placed in a water bath at 23°C and received trickle flow aeration under gold fluorescent lights at a 16h:8h light:dark cycle. Approximately 95% of the exposure solution from each beaker was renewed every 24 h and food was provided every day as 6 mg of TetraFin. Fresh aqueous solutions were prepared before each water exchange. Temperature and dissolved oxygen were monitored throughout the exposure periods. Hardness, alkalinity, and ammonia concentration were measured using commercially available kits (LaMotte, Charleston, SC) at initiation and completion of the experiments. Temperature and dissolved oxygen were monitored throughout the exposure periods. Water quality parameters including hardness, alkalinity, and ammonia were also measured using a commercially available kit from Lamotte (Chestertown, MD) at the initiation and completion of the exposures. Water quality parameters were within acceptable limits throughout the experiment: dissolved oxygen (6.5-6.9 mg/l), pH (7.4-7.6), ammonia (< 3 mg/L), hardness (100-108 ppm $CaCO_3$), alkalinity (104-116 ppm $CaCO_3$). At experiment termination, all midges from each replicate beaker were rinsed with water, blotted dry, weighed, survivors counted and stored in a 3x volume of RNA-later (Ambion) at –20 ^{o}C until used for RNA isolation.

Stocks of phenanthrene, fluoranthene, and DDT (Aldrich, Milwaukee, WI) were dissolved into acetone and added to 1 L distilled water to create solutions with nominal concentrations of 25 µg L^{-1}, 30 µg L^{-1}, and 0.2 µg L^{-1} respectively. Concentrated stocks of zinc chloride, copper chloride, and cadmium chloride (Aldrich, Milwaukee, WI) in water were added to 1 L distilled water to create solutions with nominal concentrations of 125 µg L^{-1}, 54 µg L^{-1}, and 100 µg L^{-1}. Fifty laboratory cultured, 2nd instar *C. tentans* larvae were exposed for 16 hr in 1 L distilled water in a glass beaker at room temperature for each different chemical. At the end of the exposure period, midges were removed. Midges from within the same exposure were then pooled and stored in a 3-fold volume of RNAlater^{tm} (Ambion, Austin, TX) at –20 ^{o}C until used for RNA isolation. Animals from the same exposure were pooled to minimize variations in response due to individual differences to obtain the necessary amount of RNA for quantitative analyses.

RNA isolation.

Total RNA was isolated from 100 – 500 mg tissue with an RNAgreen isolation kit using a mini-bead beater protocol and acid phenol extraction (Qbiogene). The lower phase was removed after which 20 µl of 12 M lithium chloride per 100 solution were added and RNA precipitated in a microfuge at 14 x g. RNA pellets were washed with 70% ethanol and suspended into 100 µl SAFE buffer. Contaminating DNA was removed using DNase and a DNA-free DNase removal kit (Ambion) as recommended by the supplier. RNA was quantitated using the RNA stain Ribogreen (Molecular Probes) as recommended by the manufacturer. RNA was stored at –20 ^{o}C until needed.

Gene expression analysis

Histone_H1a_2F (5'-ACCCTCGCATTTCTCAGTTT) and histone_H1a_2R (5'-GCTTGTCAGATTTCGGCTTC) primers were designed targeting *Chironomus tentans* (clone 361) histone H1a gene (GenBank accession number L29107) using the software Primer Premier V. 4 (PREMIER Biosoft International). We reverse transcribed 0.1 to 0.5 ug of total RNA into cDNA. 50 pmoles of random decamers were annealed to RNA in a 20 µl volume by heating to 85 ^{o}C for 3 minutes followed by chilling on ice. Reactions were begun with 10 µl of cDNA synthesis mix composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.4mM each deoxynucleotide triphosphate, 3 units RNase inhibitor, and 30 units M-MLV reverse transcriptase (Ambion). Reactions were placed at 42^{o}C for 2 hrs followed by 5 min at 95^{o}C to denature enzymes. Reactions were diluted to 200 µl prior to real time PCR assays. Real-time PCR assays were performed using an iCycler real-time PCR machine (BioRad). Five replicate reactions were performed for each treatment replicate. Real-time PCR reactions were composed of 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.0% Triton® X-100, 0.25 mM each deoxynucleotide triphosphate, SYBR Green I mix [final concentration of 5% dimethylsulfoxide, a 1/10,000 dilution of SYBR Green I (Molecular Probes), 1 mg ml^{-1} bovine serum albumin, 1% Tween® 20], 0.5 units Taq polymerase (Promega), 0.5 mM primers, and 2ul cDNA (representing approximately 2.5 ng total RNA) in a final volume of 20 µl. PCR conditions were 3 min at 95^{o}C, followed by 50 cycles of 95^{o}C for 30 sec, 55^{o}C for 45 sec, and 72^{o}C for1 min. Fluorescence of primer dimer products may overestimate abundance of desired PCR products. To minimize primer dimer interference, data were collected after extension at 72^{o}C during each cycle by insertion of a 10 second hold at a temperature, collection T_m, resulting in melting of primer dimers, but not desired products. Amplification of desired PCR products was determined using a melting curve from 55 ^{o}C to 95 ^{o}C in 0.4 ^{o}C increments.

Data analysis

Threshold values of fluorescence (C_t) obtained for real time reactions were analyzed for differences between experimental organisms and control organisms by determining ratios of experimental to control C_t values using the following formula: target expression relative to control = (efficiency of amplification of target PCR product) $^{(\text{control average } C_t - \text{sample } C_t)}$. Expression ratios between samples were normalized to 18S rRNA as a measure of total RNA of samples relative to controls. Relative expression values were identified as outliers and excluded from analysis if they were in excess of one standard deviation from the mean. Students 2-tailed t-test was used to assess the significance of observed differences. Pearson's ranking coefficient was used to assess relatedness. For the purposes of these experiments a value of $p < 0.05$ was used for testing of significance for all statistical tests.

Results

The effect of aqueous TNT exposures on growth and survival of midges.

The effect of TNT exposures on midges was examined from 0.5 to 2.0 mg L^{-1} TNT. Survival and growth effects were dependent upon dose and duration of exposures. Significant decreases in survival only occurred at 2.0 mg L^{-1} TNT, (Fig 5.b.9.1), consistent with a previously derived LC_{50} value of 1.9 mg L^{-1} (G. Lotufo, unpublished). Survival at 2.0 mg L^{-1} TNT decreased as length of exposures increased to 240 hrs. Growth as mass was significantly effected at 2.0 mg L^{-1} TNT at all lengths of exposure, except 1 hr.

Effects of TNT exposure on histone 1a gene expression in *C. tentans*

Histone gene expression correlates with lethal and sub-lethal endpoints in *C. tentans*. Histone 1a expression was sensitive to TNT exposure. There was no significant change in expression in 0.5 mg L^{-1} TNT with 1 to 240 hrs of exposure (Fig 5.b.9.2). Significant changes in h1a expression were observed at 24, 96 and 240 hrs of exposure to 2.0 mg L^{-1} TNT. H1a levels were decreased at 24 and 96 hrs, where reduced growth, but no change in survival was seen. A 4.4-fold increase in h1a expression over the control occurred at 240 hrs along with both reduced growth and survival.

We examined the relationship of h1a expression to concentration of TNT at 96 and 240 hrs (Fig. 5.b.9.3). Histone 1a expression over increasing TNT levels at 10 days was inversely correlated with growth and survival at 10 days (r= -0.953, -0.936 respectively). Histone 1a expression at 4 days correlated more strongly with survival and growth at 10 days (r=0.9223, 0.9329 respectively) than with survival or growth at 4-d (r=0.755, 0.0703 respectively).

Effects of other contaminants on H1a expression.

Midge larvae were stressed with low levels of phenanthrene, fluoranthene, DDT, cadmium, copper and zinc to determine whether histone 1a expression is affected by other, more common, contaminants. Concentrations used for exposures were 10-16% of 10 to 20-d LC_{50} values (Table 5.b.9.1). Low doses of DDT, phenanthrene, cadmium, and copper had significant effects on h1a expression (Table 5.b.9.1). Effects of the relative change in h1a expression normalized to the molar amount of toxicant generally reflected the relative potency of the chemicals tested as expressed by their LC_{50} values and recommended water quality criteria for the protection of aquatic organisms (USEPA 2002). Within compounds displaying significant effects, increasing levels of h1a expression was correlated with decreasing molar LC_{50} values of toxicants (Spearman r= -0.7138). Setting 1.0 Toxic Units, a dimensionless unit, to equal the respective LC_{50} values of the chemicals, allows comparison of the toxicity of compounds with differing potencies. When h1a expression is normalized to Toxic Units, expression levels weakly correlated to LC_{50} values (r=-0.597).

Discussion

We have developed a real time PCR assay for the expression of the histone 1a gene of *C. tentans* to determine the effect of chemical exposure on a protein important for cell function. Expression of h1a was found to correlate with growth and survival of midges exposed to increasing concentrations of TNT. As expected from previous studies linking histone expression to cell growth, h1a expression decreased with reduced growth in response to TNT. However, at a lethal concentration of TNT (2.0 mg L^{-1}), h1a expression increased which may reflect DNA synthesis to repair damaged cells. Levels of h1a at 4-d were correlated with growth and mortality effects at 10-d exposures. Environmental pollutants other than TNT impacted h1a expression as well. Induction levels of h1a were related to the toxicity of the compounds tested. Low levels of DDT, phenanthrene, Cd, and Cu increased histone 1a expression. The correlation of an early response with organism level effects suggests that monitoring levels of h1a expression may be useful as an early indicator of toxicity.

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6. CONCLUSIONS

The toxicity of TNT, RDX HMX, and breakdown products of TNT, in aqueous and sediment exposures.

Prior to this effort, the published information on the toxicity of sediment-associated explosive compounds was limited to one study conducted in our laboratory. Under the present research effort, we substantially expanded the knowledge base on the fate and effects of explosive compounds in the aquatic environment. We investigated the toxicity of TNT, RDX and HMX, as well as the main breakdown products of TNT, in aqueous and sediment exposures. The explosives RDX and HMX did not decrease invertebrate survival in water or sediment exposures even near water solubility limits or exceedingly high sediment concentrations (>1,000 mg/kg). Therefore, the presence of these compounds in the aquatic environment likely does not pose a serious risk to aquatic biota. The aqueous and sediment toxicity of TNT and the TNT breakdown products 2-aminodinitrotolune (2ADNT), 2,4-diaminonitrotoluene (2,4DANT) and trinitrobenzene (TNB) were compared using freshwater invertebrates. TNB was similarly toxic to TNT in both water and sediment exposures. In water exposures, nitro-reduction substantially decreased the toxicity of TNT, as the mono-aminated compound 2ADNT was less toxic than TNT. Further amination decreased toxicity much more dramatically, as the di-aminated breakdown product 2,4DANT was substantially less toxic than TNT or 2ADNT. Such dramatic differences in toxicity among compounds were not observed in exposures to spiked sediments.

Mixtures studies.

Interacting chemicals result in toxicological effects or responses that are difficult to predict based upon single chemical toxicological data. These interactions are defined as additive or non-additive (i.e., synergistic or antagonistic). Results from this investigation indicate that TNT and its daughter compounds interact additively when in a mixture. Therefore, the toxicity of a mixture to a given receptor can be reasonably predicted using single-compound toxicity derived for that receptor.

Limited information exists regarding the interaction of explosive compounds with other contaminants such as metals, PCBs, PAHs, and pesticides. Current assumptions incorporate a significant degree of uncertainty in the risk assessment process. This uncertainty is mainly due to a lack of sound-scientific information that could lead to overestimating chemical hazards and risks resulting in needless expense to the military for cleanup or remediation of military compounds. We investigated the interaction of an explosive compound (TNB) in a mixture with chemicals belonging to different classes of contaminants, more specifically the PAH phenanthrene and the heavy metal lead. Mixture interaction experiments using *C. tentans* suggest that those compounds interact non-additively to promote mortality. Although a less than additive (i.e., antagonistic) toxicological interaction was apparent, further investigation of the mixture interaction among these compounds is warranted before definitive conclusions about their mode of interaction are made.

The bioaccumulation of TNT in aquatic species

Although aqueous toxicity of TNT has been previously studied for several species of aquatic organisms, the bioaccumulation of TNT in aquatic species is poorly known. We investigated the bioaccumulation of radiolabeled TNT in two benthic invertebrates. Bioconcentration factors derived using ^{14}C-activity as a surrogate for TNT bioaccumulation (~ 100-200 g/ml) were substantially higher than values derived using HPLC-measured water and tissue sum molar concentrations of TNT and its daughter compounds (5.3 – 20.5) in this study. The difference between ^{14}C-activity and HPLC derived BCF values is likely due to the solvent-resistance of a sizable fraction (20-30%) of radiolabeled ^{14}C measured in the tissue and the presence of highly polar TNT metabolites in the extractable portion that are not quantifiable using existing standards and analytical procedures. The toxicological significance of the fraction of the body burden that does not correspond to TNT or its major degradation products remains unknown.

Toxicity metrics (e.g., median lethal concentrations, lethal body burdens) generated under this investigation were derived using laboratory experiments where clean water or sediments were spiked with chemicals at different concentrations. We attempted to validate these toxicity values using field-collected sediments. However, sediments obtained from a contaminated field site were not suitable for deriving a dose response curve using the dilution sediment approach, as concentrations in the field-collected sediments were exceedingly high in early field-collected samples or too low in later sampling attempts.

Deriving critical body residues (CBR) for benthic invertebrates.

We investigated the relationship between compound bioaccumulation and ecologically relevant toxicity endpoints, such as survival, with the objective of deriving critical body residues (CBR) for benthic invertebrates. Internal concentrations of contaminants promoting biological effects (e.g., survival and growth) are approximately constant for compounds with similar modes of action as well as among species and exposure pathways and conditions. Therefore, the CBR approach improves the interpretation of toxicity data by explicitly considering bioavailability, accumulation kinetics, uptake from all sources of exposure (e.g., sediment, food) and the effects of metabolism. As a result, risk estimates derived using CBRs as toxicity reference values are expected to carry considerably less uncertainty than those derived using water or sediment concentrations expected to promote biological effects. Critical body residues were determined for *C. tentans* in aqueous exposures to TNT, 2ADNT and 2,4DANT. CBRs, expressed as the sum molar concentrations of chemically detected nitroaromatics, were relatively constant for all compounds, ranging from 0.02 to 0.12 µmol/g. Therefore, it is likely that TNT and its major breakdown products act by similar modes of action in aquatic invertebrates, further supporting the assumption of additivity for the interaction of those compounds.

Development of genetic assays for assessing exposure to explosives and metals.

Site assessment and risk analysis efforts are often hampered by lack of sufficient data and unnecessarily strict cleanup levels. Rapid, inexpensive bioassays for chemical effects will enable screening of larger numbers of samples to establish realistic cleanup levels and delineate contamination hotspots. Using a common sediment bioassay organism, *L. plumulosus*, we have isolated genes useful in assessing exposure to bioavailable chemicals in contaminated sediments and waters. Prior to this work virtually no genetic information existed for *L. plumulosus*. We have isolated and identified genes involved in several important biological pathways. A cDNA similar to pyruvate dehydrogenase E1 component, beta subunit was isolated. The reactions of the pyruvate dehydrogense complex serves to interconnect the metabolic pathways of glycolysis, gluconeogenesis and fatty acid synthesis to the tricarboxylic acid cycle, the principal source of adenosine triphosphate for cellular energy. As a result, changes in regulation of pyruvate dehydrogense complex can severely effect growth and survival. A cDNA was isolated for 26S protease subunit, a component of the principle pathway for degradation of cellular proteins. A cDNA was isolated that was similar to QM protein. QM, a novel gene that was first isolated as a putative tumor suppressor gene from a human Wilms' tumor cell line, may have an important biological role associated with development. Genomic clones were isolated for Mn-superoxide dismutase, an enzyme involved in controlling oxidative stress and oxygen toxicity by converting the superoxide radical to less dangerous forms. Several families of mobile genetic elements were described for the first time in this work. Mobile genetic elements can cause mutations and chromosomal rearrangements. In the freshwater midge, *Chironomus tentans*, we show that a protein involved in chromosome structure and gene regulation is itself effected by exposure to contaminants and may serve as a useful biomarker for exposure. Each of the genes isolated in this work reflect important pathways that can be affected by toxicants, therefore these assays are attractive tools for assessing effects of many different chemicals on *L. plumulosus* and *C. tentans*

Relationship of genetic assays to whole organism and population levels measures.

We have developed several rapid assays to assess effects on important genes present in *L. plumulosus* and *C. tentans*. By correlating results from these assays to whole organisms responses, we have related responses on the genetic level to the whole organism level. Using these assays, we have been able to correlate expression of certain genes to sublethal bioaccumulation of TNT and lead. Assays were also found to correlate with CBRs of lead and phenanthrene. By assaying the effects of chemicals on the specific genes, mechanistic inferences can be made about toxic modes of action. TNT exposures induced expression of several genes and mobilized several mobile genetic elements. Since mobile genetic elements create heritable changes in genomes (i.e. mutations, deletions, nonhomologous recombination), mobilization of transposable elements will directly impact future generations and populations. Bioaccumulation of lead was directly related to levels of a superoxide dismutase. These assays should also be useful for short-term sediment screening protocols, in addition to aquatic exposures since animals can be recovered from exposure sediments. Sufficient sample was obtained from 20 amphipods to perform assays. This suggests that many more samples could be screened at a smaller scale (5 organisms) to create very high throughput screening protocols. Once protocols have been established, these gene assays should be very cost effective for screening sediment and water samples for toxicity.

7. TRANSITION PLAN

Technical results from this research have been transitioned to managers by publication of peer-reviewed papers, presentations at scientific meetings, and through risk assessment models being developed within the Army. Our research addressed a current and future challenge for the conduct of environmental risk assessments at Army and DOD sites. We generated a wide body of information on the toxicity, mixture interactions, bioaccumulation and critical body residues of explosive compounds. This body of information will be relevant for conducting environmental risk assessments at Army and DOD sites contaminated with explosive compounds. Data generated during this project will also be used to develop toxicity models within the Army Risk Assessment Modeling System (ARAMS). The ARAMS is being designed as an integrated contaminant fate and transport and effects modeling system for characterizing contaminant risk on Army sites. Extensive interaction with regulatory agencies during the development of ARAMS is helping to ensure regulatory acceptance of the modeling system and the tools it implements. The modeling system incorporates exposure modeling in various media (aquatic and terrestrial), toxicological effect data for both human and ecological endpoints, and determines risk while including routines for describing uncertainty. Our research generated relevant information on the relationship between an ecologically significant toxicological effect (i.e., survival) and internal chemical concentrations (i.e., whole organism body burden) to a diversity of Army-relevant compounds and biological receptors. Critical body residue data will be incorporated within the Environmental Residue-Effects Database (ERED, http://www.wes.army.mil/el/ered/index.html), which is housed at the Environmental Laboratory at ERDC. This database has been integrated with ARAMS as a source of toxicity data. We are continuing to enhance capabilities to conduct population-level risk assessments. Under the Hazard/Risk focus area of the Installation Restoration Research Program, modeling tools are being developed to accomplish population level assessments using the kinds of data generated during this study. Gene assays developed here are being assessed for utility as tools for screening sediments containing non-military-specific contaminants of concern (i.e. other PAHs, PCBs, metals, and pesticides) using research funds from the Army Corps Engineers Long-term Effects of Dredging Operations research program. Further funding to apply this research to field contaminated samples and understand applicability of the genetic assays to screen for toxicity of water and sediment contaminated with military-unique compounds will be sought under the ESTCP program to ensure application to other installations. There is strong recognition within the scientific and risk assessment community of the importance of this work, and we are currently seeking funding to perform a field validation of laboratory data to fully characterize both chemical and site-specific variability of chemical toxicity and mixture interactions.

8. RECOMMENDATIONS

We generated a wide body of information on the toxicity, mixture interactions, bioaccumulation and critical body residues of explosive compounds in aquatic invertebrates. This body of information will be relevant for conducting environmental risk assessments at Army and DOD sites contaminated with explosive compounds. The toxicity metrics (e.g., median lethal concentrations, lethal body burdens) generated under this investigation were derived using laboratory experiments where clean water or sediments were spiked with chemicals at different concentrations. The use of toxicity metrics derived from laboratory exposures using spiked sediment or water as toxicity reference values for effects assessment in risk assessments of explosive compounds in aquatic systems should to be validated prior to wide-scale use and application. Because of difficulties in predicting the bioavailability of explosive compounds in sediment, toxicity metrics expressed as concentration in environmental matrices (sediment or water) may not be appropriate to predict toxicity from exposures to field-collected sediments. Toxicity metrics derived using tissue concentrations (i.e., critical body burdens or CBRs) determined using spiked sediments are more likely to be reliable toxicity reference values. The validation of this approach using a wide variety of field-contaminated sediments as well as *in situ* toxicity and bioaccumulation exposures at contaminated sites is highly recommend as a follow-up investigation to this project.

The transition of the gene assays as screening tools will require determining the specificity of the assays in relation to a wider range of contaminants, both in water and in sediment. In addition, correlation of genetic markers to toxic responses and effects should be validated with field-contaminated samples. We have developed several rapid genetic assays that correlate with body burden. To apply this rapid screening to other species of concern will require development of species-specific gene assays.

APPENDIX A:

Appendix A1: Peer reviewed publications:

PUBLISHED STUDY 1 - Lotufo et al., 2001.

Lotufo, G.R.; Farrar, J.D.; Inouye, L.S.; Bridges, T.S.; Ringelberg, D.B. 2001. Toxicity of sediment-associated nitroaromatic and nitrocylamine compounds to benthic invertebrates. *Environ Tox Chem* 20: 1762-1771. see attached PDF file "lotufo 2001.pdf"

PUBLISHED STUDY 2 - Fredrickson et al., 2001.

Fredrickson, H., Perkins, E., Bridges, T., Tonucci, R., Fleming, J., Nagel, A., Diedrich, K., Mendez-Tenorio, A., Doktycz, M., Beattie, K. (2001). Towards environmental toxicogenomics: development of a flow-through, high-density DNA hybridization array and its application to ecotoxicity assessment. *The Science of the Total Environment*, 274:137-149 see attached PDF file "Fredrickson 2001.pdf"

PUBLISHED STUDY 3 - Steevens et al., 2002 .

Steevens J.A., Duke, B.M., Lotufo, G.R., Bridges, T.S. 2002. Toxicity of the explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in sediments to *Chironomus tentans* and *Hyalella azteca*: Low-dose hormesis and high-dose mortality. *Environ Tox Chem* 21: 1475-1482. see attached PDF file " Steevens 2002.pdf"

PUBLISHED STUDY 4 - Perkins and Lotufo 2003.

Perkins, E., Lotufo, G. (2003). Playing in the mud- using gene expression to assess contaminant effects on sediment dwelling invertebrates. *Ecotoxicology* (in press).

Title: Playing in the mud- using gene expression to assess contaminant effects on sediment dwelling invertebrates.

Running title: Bioavailability, toxicity, and gene expression in benthos

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Summary

Bioaccumulation and toxicity tests using benthic invertebrates such as the estuarine amphipod Leptocheirus plumulosus are typically used to assess the ecological risk associated with contaminated sediments Despite their ecological and regulatory importance, little is known about such species at the genetic level. To begin understanding cellular and genetic responses of L. plumulosus to contaminants, we isolated several of their genes and developed quantitative assays to measure the effects of water exposures to 2,4,6-trinitrotoluene (TNT) and phenanthrene on gene expression. Real-time polymerase chain reaction (PCR) assays demonstrated that the expression of the genes for actin and a retrotransposon, Hopper, was dependent on the exposure and tissue concentrations of those chemicals. Our data suggests that exposure to the explosive 2,4,6-trinitrotoluene and phenanthrene may induce movement of Hopper resulting in unexpected genotoxic results.

Keywords: Bioaccumulation, 2,4,6-trinitrotoluene, polyaromatic hydrocarbon, real time PCR, retrotransposon

Introduction

The United States Army Corps of Engineers (USACE) is actively engaged in the development of rapid and inexpensive screening tools for assessing adverse effects of sediment-associated contaminants. To maintain proper navigation, the USACE manages the dredging and disposal of large volumes of sediments potentially contaminated with a variety of chemicals (e.g., polyaromatic hydrocarbons, polychlorinated biphenyls, and heavy metals). In addition, the U.S. Army has some 40 installations requiring cleanup of soils and sediments contaminated with explosives compounds such as trinitrotoluene (TNT) and lead.

Benthic invertebrates are typically used in water and sediments toxicity tests for assessing the potential detrimental ecological effects posed by contaminated sediments (Emery *et al.*, 1997; USEPA/USACE, 1998; USEPA, 2001). Chronic sublethal tests are expected to be more sensitive indicators of the detrimental environmental effects of low to moderate levels of contamination due to the longer exposure duration and the measurement of sublethal responses such as growth and reproduction. However, chronic toxicity tests are lengthy (weeks to months), labor-intensive, and typically expensive (thousands of dollars per sample) (Gray *et al.*, 1998). Rapid and inexpensive methods are needed for screening large numbers of sediment samples containing moderate to low levels of chemicals.

Gene-based assays for exposure effects in a benthic invertebrate

The sediment-dwelling amphipod Leptocheirus plumulosus is routinely used for toxicity testing of estuarine and marine sediments (USEPA, 2001) Rapid, gene-based assays that correlate to whole organism effects in L. plumulosus could be extremely useful as sediment toxicity and contaminant bioavailability screening tools. We have designed several real-time polymerase chain reaction assays (real-time PCR) assays to monitor gene expression in L. plumulosus. Real-time PCR assays are essentially modifications of PCR assays where product accumulation is monitored at the end of each cycle (for review see Bustin, 2000). Many different approaches have been developed to monitor this binding (e.g., Taqman assays, molecular beacons, and hybridization probes). The simplest approach uses the dye SYBR Green I (Bustin, 2000) whose fluorescence is 100 times greater when bound to double stranded DNA. As double stranded PCR product accumulates, more SYBR Green I is bound, resulting in successively higher levels of fluorescence. The concentration of the target cDNA (representing mRNA isolated from the test organism) can then be determined from the point at which fluorescence emerges from background by comparison to a control or known concentration standards.

Linkage of sub-lethal gene expression with lethal and sub-lethal effects in L. plumulosus

Linkage of gene expression assessment with whole organism effects is essential in the development of real-time PCR assays as bioindicators of effects using real-time PCR. For screening tools to be useful, evidence is required that gene expression assays can be correlated with toxicity observed at the whole organism scale. Using PCR primers targeting evolutionarily conserved genes we have isolated genes from L. plumulosus for actin, superoxide dismutase, pyruvate dehydrogenase and a mobile genetic element, Hopper, a putative non-long terminal repeat retrotransposons (Perkins, in preparation).

In 4-day water exposures of L. plumulosus to TNT and phenanthrene, gene expression, survival and tissue residues were assessed concomitantly. Real-time PCR assays for actin, a house keeping gene involved in cytoskeleton structure and mobility, and Hopper gene expression normalized to 18S rRNA were used to demonstrate the potential for linking gene expression to the bioaccumulation and survival.

Leptocheirus plumulosus exposed to TNT exhibited a dramatic decrease in survival at exposures equal to or greater than a mean body residue of 726 nmol g^{-1} wet wt (Fig 1). TNT dramatically affected the expression of actin and Hopper. Increased expression levels of actin, and Hopper, normalized to 18S ribosomal rRNA transcript levels, were associated (Pearson product moment correlation r=0.861 and 0.914, respectively) with increasing sublethal levels (0 - 500 nmol g^{-1} tissue) of TNT in the tissue (Fig 1). Increased gene expression with increasing sublethal body burden was also observed in other genes such as a 60S ribosomal protein and b4, a cDNA with unknown function, (data not shown). A general increase in gene expression at low levels of TNT is consistent with observations of increased growth in L. plumulosus chronically exposed to low concentrations of TNT (Green *et al.*, 1999).

Phenanthrene promoted exposure effects in L. plumulosus substantially different from those observed with TNT (Fig 2). An increase in Hopper expression was observed only at 0.011 mg L^{-1} phenanthrene, the lowest concentration at which survival was significantly affected. At concentrations greater than 0.011 mg L^{-1}, Hopper expression dropped to one-tenth of control level. No effect occurred on actin expression in phenanthrene exposed L. plumulosus. Unlike TNT exposures, Hopper expression levels did not correlate with phenanthrene tissue residue (Pearson product moment correlation r=-0.033, n=15).

Not all contaminants tested had significant effects on gene expression. Exposure of L. plumulosus to concentrations of lead from 0 to 1.51 mg L^{-1} had no significant effect on Hopper or actin gene expression. Correlation of expression effects with either body residue (TNT) or exposure concentrations (phenanthrene) may be reflective of different mechanisms of toxicity or chemical transformations. For both TNT and phenanthrene, high levels of Hopper gene expression occurred at or prior to significant effects on survival being observed, suggesting that expression of this gene could be indicative of sub-lethal exposure effects. At levels where significant mortality occurs, Hopper and actin expression decrease to normal or below. If these assays are used to infer toxicity or exposure effects, care must be taken to incorporate mortality effects. However, basing determinations of toxicity solely on gene expression data from exposures at or past lethal concentrations may lead to false conclusions of lower toxicity. Further tests varying exposure period and with lower concentrations will determine how well effects in chronic exposures relate to effects on gene expression determined in short-term exposures.

While changes in gene expression of biomarkers do not directly imply deleterious effects, changes in expression and number of Hopper retrotransposons may directly cause significant long-term effects on populations since retrotransposons are most active in germ-cell lines (Zhao and Bownes, 1998; Stuart *et al.*, 2001). Transcription levels of reverse transcriptases are thought to be the rate-limiting step in transposition (Mathais and Scott, 1993). Therefore, increased expression of Hopper could lead directly to genotoxic effects due to increased retrotransposition. We have found the number of Hopper copies to increase 3.2-fold in L. plumulosus genomic DNA after 4-days exposure to TNT (Perkins, in preparation). Retrotransposons similar to Hopper have been documented to cause a wide range of effects on genetic diseases and cancer (Miki *et al.*, 1992, Hagan and Rudin, 2002), double strand DNA break repair (Teng *et al.*, 1996; Tremblay *et al.*, 2000; Morrish *et al.*, 2002), expression of adjacent genes (Speek, 2001), and interruption of genes creating pesticide resistant insects (Gahan *et al.*, 2001).

Conclusion

We have isolated a number of genes from widely employed sediment bioassay organism, Leptocheirus plumulosus, including actin and several putative mobile genetic elements. Complex patterns of gene expression are observed in L. plumulosus exposed to different contaminants. Gene expression of actin and a putative retrotransposon, Hopper, was directly related to bioaccumulation of TNT prior to the onset of significant mortality. Phenanthrene effects on Hopper resembled a threshold effect where increased expression of was observed at phenanthrene levels causing significant mortality, but higher levels of exposure significantly reduced Hopper expression. Genetic markers such as Hopper could serve as measures of genotoxic potential.

Acknowledgements

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Figure 1.
Gene expression in L. plumulosus as a function of TNT tissue residue. Solid circles represent levels of actin and open circles levels of Hopper gene expression in exposures. Expression was normalized to 18S rRNA copies and is relative to unexposed controls. Error bars indicate real-time PCR assay replicates for each exposure replicate. The dashed line represents the number of survivors after four days exposure out of 100 individuals.

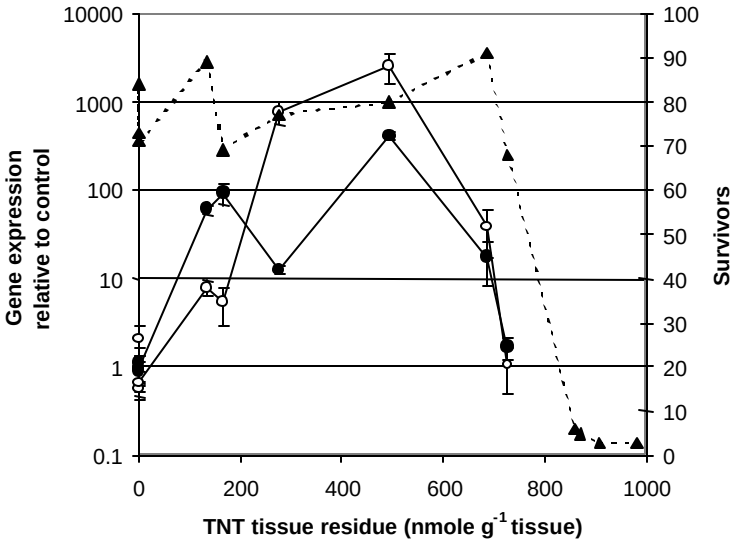
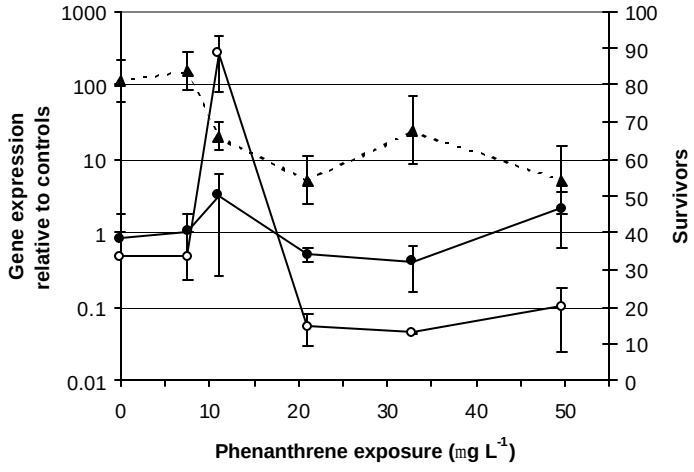


Figure 2.
Gene expression in L. plumulosus as function of phenanthrene water concentration. Solid circles represent levels of actin and open circles levels of Hopper gene expression in exposure replicates. Expression was normalized to 18S rRNA copies and is relative to unexposed controls. The dashed line represents the number of survivors after four days exposure out of 100 individuals.



PUBLISHED STUDY 5 - Perkins et al., 2003.

Perkins, E., Lotufo, G., Farrar, J.D. Chemical stress induces transposition of a retrotransposon in a benthic amphipod (submitted to Nature Genetics, March 2003)

Chemical stress induces transposition of a retrotransposon in a benthic amphipod.

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SUMMARY

Transposable elements (TE) are thought to play a significant role in adaptation and evolution of genomes via insertions, deletions and nonhomologous recombination[1]. Mobilization of TEs would permit rapid adaptive responses to environmental shocks [2,3]. Yet, with the exception of plants and microbes, few examples exist where transposition occurs in response to chemical stress. We have isolated several genes from *Leptcheirus plumulosus* similar to retrotransposons and transposons. *L. plumulosus* must adapt to many different chemicals as it feeds on sediment particles in estuaries from New England to Northern Florida in the United States [4]. Transcripts of two non-LTR retrotransposons and two transposons accumulated with exposure to increasing amounts of 2,4,6-trinitrotoluene (TNT). Hopper expression also increased at lowest observable effect concentrations for survival in phenanthrene and lead acetate exposures. Sublethal levels of TNT increased hopper genomic copy numbers 3.2-fold indicating hopper is an actively transposing element. Increased transposition correlated with changes in genomic DNA amplified fragment length polymorphisms. Mobilization of TEs in response to chemical stress provides *L. plumulosus* an opportunity for rapid genome remodeling and adaptation to an environmental stressor.

We isolated several different TEs from *L. plumulosus*. Eighteen different clones grouped into at least 12 subgroups of related sequences were isolated with high degrees of similarity to retrotransposons and transposons (Table 1). Several of these clones were also similar to conserved reverse transcriptase protein domains of retrotransposons and conserved transposase domains of transposons (Table 1). Sequence similarities and phylogenetic tree reconstructions indicate that *L. plumulosus* possesses at least three of the five known distinct groups of TEs, excision-based DNA transposons related to the Tc1/Mariner family, replication based long terminal repeat (LTR) retrotransposons related to the Gypsy/Ty-3 family, and replication based non-LTR retrotransposons (Table 1). The clones *tank*, *tank.1*, *stealth*, *stealth.1*, LpTc1, and LpTc1.1 are related to the Tc1/Mariner DNA transposon family (Fig. 1). The clone ranger is related to the Gypsy/Ty-3 LTR retrotransposon family. Non-LTR retrotransposons have been separated into 11 clades based upon reverse transcriptase domain similarities [6]. The clones *dredger*, *hopper*, *hopper.1*, *hopper.2*, *hopper.3*, LpRt1, and LpRt1.1 are related to the Jockey clade (Fig. 2). The clone LpRt is related to the RTE clade. The clones LpRt2, LpRt3, LpRt4, and LpRt5 are related to the CR1 clade (Fig. 2).

Studies of plants and humans suggest that TEs are inducible by stress [6-8]. We tested the hypothesis that the probable TEs isolated from L. plumulosus would be affected by exposures to chemical stress. We used semiquantitative, real time PCR assays to assess the effect of TNT contamination on TEs present in *L. plumulosus*. The expression of four clones were examined, the LTR retrotransposon *ranger*, the non-LTR retrotransposon *hopper*, and the transposons stealth and *tank*. A fifth clone, *hopper.2*, was examined that contains a 152-bp deletion in the reverse transcriptase coding region, but is otherwise 95.6% identical to *hopper*.

Adult amphipods were exposed to 0, 0.1, 0.25, 0.5, 1.0 and 2.0 mg L^{-1} TNT in an attempt to induce TEs. After 4-days of exposure to TNT, we observed significant decreases in survival at tissue residues greater than 726 nmol g^{-1} wet wt (Fig. 3). TNT degraded in solution over time resulting in different exposures on a mg L^{-1} TNT basis (data not shown) therefore exposure to TNT was measured as the total amount of chemical originally in the form of TNT entering an animal (tissue residue). Transcript levels from all TEs, except *hopper.2*, increased with sublethal doses up to 500 nmol TNT g^{-1} tissue (Spearman ranking $r = 0.927$, $P=0.0067$) of TNT in the tissue (Fig 3). At TNT doses approaching lethal concentrations, expression of *ranger* and *hopper* reverse transcriptases and expression of stealth and tank transposases decreased to basal levels. No expression of the reverse transcriptase of *hopper*.2 was observed in control or exposed populations. This observation is consistent with predictions that *hopper*.2 is a defunct copy of hopper due to a deletion in the reverse transcriptase domain.

To determine if other compounds could affect TEs of *L. plumulosus*, we examined the effects of lead and the polyaromatic hydrocarbon phenanthrene on hopper and actin. Adult amphipods were exposed to 0, 7, 11, 22, 34 and 50 ug L^{-1} phenanthrene. After 4-days of exposure, *hopper* transcripts increased at the lowest concentration at which survival was significantly affected (18.1% reduced), 0.011 mg L^{-1} phenanthrene (Fig. 4). At higher concentrations, hopper levels dropped to one-tenth of controls. Unlike TNT exposures, *hopper* expression levels did not correlate with phenanthrene tissue residue (Spearman r =-0.6571, p = 0.175). 4-day exposures of amphipods to 0.03, 0.12, 0.16, 0.74, and 1.51 mg L^{-1} lead resulted in a significant decrease in survival (16.7%) only at the highest level of lead tested (Fig. 5). Notably, expression of *hopper* was 2.14 ± 0.48 (Student's t-test p=0.0124) higher than controls at 1.51 mg L^{-1} lead. For TNT, phenanthrene, and lead high levels of TE transcripts occurred at or immediately prior to significant effects on survival. These observations suggest that high levels of stress, approaching lethality, may be related to activation of TEs rather than a chemical specific response.

Transcription levels of reverse transcriptases are thought to be the rate-limiting step in transposition of retrotransposons such as human LH1 [9]. High frequencies of I element mediated insertions and rearrangements in Drosophila melanogaster are caused by a five-fold increase in I element expression [10, 11]. Therefore we hypothesized that increased expression of *hopper* would lead to increased replicative transposition. To test whether increased transcription resulted in increased transposition and integration of more copies of *hopper*, we exposed five replicate populations of 20 juvenile *L. plumulosus* to sublethal levels of TNT (0.5 mg L^{-1}) for four days. Individuals from within a replicate were pooled, DNA extracted, and treated as one replicate population. We compared genomic copy numbers of *hopper* in exposed and unexposed replicate populations using quantitative, real time PCR (Fig. 6). The average copy number of hopper in TNT exposed populations was 3.2-fold higher than control populations (Student's t-test p=0.0386). This data indicates that chemical stress can mobilize retrotransposons within *L. plumulosus*.

Mobilization and insertion of TEs has many effects. Active transposition has long-term effects on populations since many retrotransposons are active in germ-cell lines [12]. A wide range of effects have been documented including buffering of genome from chromosomal degradation by expansion of telomeric ends [13], involvement in genetic diseases and cancer [14], double strand DNA break repair [15], gene regulation [16] and interruption of genes leading to pesticide resistant insects [17]. Each of these effects are expected to impact the structure of the genome. We examined the genomic DNA of populations exposed to TNT to determine whether transposition, as evidenced by increased copy numbers in the case of hopper, correlated with changes in the genome. We used amplified fragment length polymorphism analysis (AFLP) to detect changes in genomic DNA isolated from exposed and control populations [18].

As copy numbers of hopper increased in a population, the number of AFLP fragments observed decreased (Pearson r = -0.769, P value = 0.0094, Fig. 6) and AFLP profiles changed (data not shown). Correlation of hopper copy number and AFLP fragment number indicates significant changes occurred in genomes where active transposition has occurred. DNA damage may also be involved in alteration of AFLP patterns since randomly amplified polymorphic DNA patterns change with damage [19] and TNT can cause DNA damage[20].

Our findings indicate that the genome of *L. plumulosus* contains at least three different families of TEs. Four TEs were found to be transcriptionally active and at least one, hopper, actively transposes under chemical stress. Although we tested only a limited number of chemicals and one metal, hopper transcript levels appear to be more closely related to the level of stress or lethality rather than specifically to any of the three toxicants. TNT, lead, and polyaromatic hydrocarbons such as phenanthrene can cause DNA damage [20-22] and retrotransposons have been implicated in DNA repair [15] leading us to suggest that *hopper* and other retrotransposons may be responding to genotoxic effects such as DNA lesions. Our data is consistent with McClintock's genome shock theory[2] where environmental shock, here near lethal concentrations of contaminants, would result in genomic shock, here mobilization of TEs, providing *L. plumulosus* an opportunity for rapid genome remodeling and adaptation to an environmental stressor.

Materials and methods

Exposure media.

A TNT spiking stock was prepared for each water treatment by combining ^{14}C-labeled TNT to non-radiolabeled TNT in the appropriate volume of acetone. Radiolabeled trinitrotoluene (^{14}C-TNT, 23.6 Ci/mol, 99% radiochemical purity) was purchased from New England Nuclear Research Products (Boston, MA). Non-radiolabeled TNT (99% purity) was purchased from Chem Service (Westchester, PA, USA). Exposure water was prepared by spiking 0.5 ml of TNT acetone stock to each liter of artificial seawater. The target radioactivity in all exposure water treatments was 5,000 dpm/ml. The target TNT concentration in water treatments were 0.25, 0.5, 1.0, 3, and 6 mg L^{-1}; actual mean measured concentrations for experiment start and day three were 0.38, 0.66, 1.39, 3.79, and 7.39 mg L^{-1}. The control treatment consisted of a 0.5 ml/L acetone solution. A phenanthrene (PHE) spiking stock was prepared for each water treatment by combining ^{14}C-labeled PHE to non-radiolabeled PHE in the appropriate volume of acetone. Radiolabeled trinitrotoluene (^{14}C-PHE, 23.6 Ci/mol, 99% radiochemical purity) was purchased from New England Nuclear Research Products (Boston, MA). Non-radiolabeled PHE (99% purity) was purchased from Chem Service (Westchester, PA, USA). Exposure water was prepared by spiking 0.5 ml of PHE acetone stock to each liter of artificial seawater. The target radioactivity in all exposure water treatments was 100 dpm/ml. Target PHE concentrations in water treatments were 0.007, 0.021, 0.032 and 0.047 mg L^{-1} ; actual mean measured concentrations for experiment start and day three were 0.0074, 0.011, 0.021, 0.033, and 0.050 mg L^{-1}. The control treatment consisted of a 0.5 ml/L acetone solution. For lead, exposure water solutions were preparing by adding the appropriate volume of a concentrated lead chloride aqueous (3,000 mg L^{-1}) solution to artificial seawater. The target lead concentration in water treatments were 0.1, 0.2, 1.0, and 2 mg L^{-1}; actual mean measured concentrations for experiment start and day three were 0.03, 0.12, 0.16, 0.74, and 1.51 mg L^{-1}.

Aqueous exposures.

Adults (3-8 mg) laboratory cultured *Leptocheirus plumulosus* [31] were exposed aqueous solutions of TNT, phenanthrene and lead for 4 days. Amphipods were exposed in 4-L beakers. Four beakers were used for each treatment. Each exposure beaker received 100 organisms. Beakers were placed in a water bath at 23°C under gold fluorescent lights at a 16:h-8h light:dark cycle. Beakers were not aerated and no food was provided. The exposure solution from each beaker was fully renewed every 24 h. Exposure water was sampled for radioactivity determination (TNT and PHE) and chemical analysis (Pb) at the beginning of the experiment and daily thereafter before and after each exposure water renewal for monitoring compound concentration throughout the exposure period and to determined compound volatilization and degradation following the 24-h period preceding each exposure solution exchange event. One milliliter of water was transferred to 12-ml scintillation cocktail (3a70b; Research Products International) and ^{14}C-activity was quantified by liquid scintillation counting (LSC) on a Tricarb Liquid Scintillation Analyzer (Packard Instruments). At exposure termination, 3-5 amphipods were blotted dry, weighed, and transferred to 12-ml scintillation cocktail, and analyzed for radioactivity as described above. Remaining amphipods from each beaker were rinsed with water, blotted dry, weighed, and stored in a 3x volume of RNA-later (Ambion) at –80 ^{o}C until used for chemical and genetic analysis.

RNA isolation.

Total RNA was isolated from 200 – 500 mg tissue (20-100 organisms) with an RNA green isolation kit using a mini-bead beater protocol and acid phenol extraction (Qbiogene) with the following modifications: after precipitation of RNA from aqueous solution with isopropanol precipitation solution rather than pelleted RNA, two liquid phases were observed. The lower phase was removed after which 20 ul 12 M lithium chloride per 100 solution were added and RNA precipitated in a microfuge at 14 x g. RNA pellets were washed with 70% ethanol and suspended into 100 ul SAFE buffer. Contaminating DNA was removed using DNase and a DNA-free DNase removal kit (Ambion) as recommended by the supplier. RNA was quantitated using the RNA stain Ribogreen (Molecular Probes) as recommended by the manufacturer. RNA was stored at –20 ^{o}C until needed.

Consensus-Degenerate PCR .

PCR primers used for isolation of conserved gene fragments are described in Fredrickson et al [23]. Briefly, alignments present in the protein family database, Pfam [24], were used as input to the program MAKEBLOCK and the consensus-degenerate primer design program, CODEHOP [25]. Primers were checked for uniqueness against the GenBank sequence database using the GCG Wisconsin package program FindPatterns (accelrys). Genomic DNA from *L. plumulosus* was isolated from 200 mg tissue with FastDNA Kit (Qbiogene) using a mini-bead beater protocol. PCR reaction mixtures contained 75 ng of genomic DNA, 0.2 µM of dNTPs, 0.4 µM of primers, 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 40mM $MgCl_2$, 1 unit Taq DNA polymerase (Stratagene) in a total volume of 25 ul. The reaction mixture was heated 95ºC for 5 min and followed by 30 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1min. The final cycle was an extension at 72ºC for 10 min. PCR products were separated in a 20 x 20 cm, 7% non-denaturing Long Ranger^{tm} hydrolink gel (Cambrex). DNA was visualized after staining with SYBR Green I dye (Molecular Probes), using 310 nm transillumination and a ChemiImager 4000 CCD camera system (Alpha Innotech.)

Cloning and sequencing of PCR fragments.

Bands were excised from gels using a razor blade or the tip of an 18-gauge needle. PCR fragments were purified by heating the gel slice with 100 ul dH_20 at 100 ^oC for 5 min. The heated gel slice was removed by centrifugation and the PCR fragment purified from solution by precipitation with 1 ul Pellet Paint mussel glycogen co-precipitant (Novagen), 1/10 volume 3M Sodium Acetate (pH 5.2) and 3 volumes ethanol. Precipitated DNA was resuspended into 10 ul TE. 1 ul isolated PCR fragment was then reamplified using the primer set used in the original PCR reaction from which the band was isolated. Successful reamplifications were purified using a QIAquick PCR Purification Kit (Qiagen Corp.) according to manufacturer's protocols and cloned into the PCR fragment cloning vector pCR2.1-TOPO per manufacturers recommendations (Invitrogen). Plasmid DNA was isolated and cleaned using a QIAprep Spin Miniprep Kit (Qiagen). Clones were sequenced using an ABI 3100automated capillary DNA sequencer (Applied Biosystems) and Big Dye Terminator V. 3 fluorescent dye terminator cycle sequencing kits (Applied Biosystems) as recommended. Prior to sequencing, unincorporated dyes were removed using Centri-Sep purification columns (Princeton Separations). Resulting sequences were identified by comparison of all possible reading frames to known sequences in the National Center for Biotechnology Information's non-redundant protein database using the programs BLASTX with BEAUTY post-processing provided by the Human Genome Sequencing Center, Baylor College of Medicine [26].

Gene expression analysis.

We designed real time PCR primers for 6 genes having high similarity to reverse transcriptases, or transposase sequences using the software Primer Premier V. 4 (Premier Biosoft International). The primer sequences are: ranger (forward: CTGGTGGTTGCAGTCTTGTG, reverse: TGGTATTCGTCCCTGTATCG), hopper.2 (forward: TTGAGGCCAGGATGGATG, reverse: CCTATTCTGTCTAGGGTGTACGAG), hopper (forward: CGCAGCTTATGGAGCAATC, reverse: TTTCTGCCTCCCAACCAA), tank (forward: TTTGCTATTTGGTCCATCTT, reverse: CGTTTGAGCCTGAGTGCC), and stealth (forward: ATGGAACCCTCGTCACTC, reverse: CGTGCGTTCATAACTGTCT). QuantumRNA universal 18S rRNA primers (Ambion) were used to determine abundance of 18S rRNA. We reverse transcribed 0.1 to 0.5 ug of total RNA into cDNA. 50 pmoles of random decamers were annealed to RNA in a 20 ul volume by heating to 85 oC for 3 minutes followed by chilling on ice. Reactions were begun with 10 ul of cDNA synthesis mix composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.4mM each deoxynucleotide triphosphate, 3 units RNase inhibitor, and 30 units M-MLV reverse transcriptase (Ambion). Reactions were placed at 42°C for 2 hrs followed by 5 min at 95°C to denature enzymes. Reactions were diluted to 200 ul prior to real time PCR assays. Real-time PCR assays were performed using an iCycler real-time PCR machine (BioRad). Five replicate reactions were performed for each treatment replicate. Real-time PCR reactions were composed of 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.0% Triton® X-100, 0.25 mM each deoxynucleotide triphosphate, SYBR Green I mix [final concentration of 5% dimethylsulfoxide, a 1/10,000 dilution of SYBR Green I, 1mg ml^{-1} bovine serum albumin, 1% Tween® 20], 0.5 units Taq polymerase (Promega), 0.5 mM primers, and 2ul cDNA (representing approximately 2.5 ng total RNA) in a final volume of 20 ul. PCR conditions were 3 min at 95°C, followed by 50 cycles of 95°C for 30 sec, 55°C for 45 sec, and 72°C for1 min. Fluorescence of primer dimer products may overestimate abundance of desired PCR products. To minimize primer dimer interference, data was collected after extension at 72°C during each cycle by insertion of a 10 second hold at a temperature, collection T_m, resulting in melting of primer dimers, but not desired products. Amplification of desired PCR products was determined using a melting curve from 55 °C to 95 °C in 0.4 °C increments.

Quantification of hopper DNA and RNA.

We tested the effect of sublethal levels of TNT on hopper genomic copy number and expression levels by exposing five replicate populations of 20 juvenile L. plumulosus for four-day to 5 mg L^{-1} TNT in parallel with five replicate control populations. We pooled individuals within a replicate population and extracted genomic DNA from each population using FastPrep mini-bead beater (Qbiogene) and TRIzol reagent (Invitrogen) as recommended by the manufacturer. Abundance of hopper in genomic DNA was determined by real time PCR and levels between samples were normalized to relative amounts of L. plumulosus superoxide dismutase gene, SOD2 (forward primer: TTTTCACAAGCCAGCACCAG, reverse primer: CGGCGAGTTTGAGCCATAA). Real time PCR conditions for genomic DNA were as described above.

AFLP analysis.

AFLP analysis was performed using an AFLP plant mapping kit (Applied Biosystems) and an ABI 3100 capillary sequencer (Applied Biosystems) as recommended by the manufacturer. AFLP products amplified using the selective primer combination MseI-CAA and EcoRI-ACT were sized with an internal size standard, Rox dye-labeled Genescan 500 (Applied Biosystems). AFLP patterns were captured and peaks were assigned base pair sizes using GeneScan analysis software (Applied Biosystems). Genotyper software (Applied Biosystems) was used to assemble a data matrix scoring for presence or absence of a band from 75 to 500 bp in size.

Phylogenetic reconstruction.

We compared the L. plumulosus reverse transcriptase and transposase open reading frame translations with reverse transcriptase or transposase domains of highly similar or those representing different clades of each domain. We used quartet-based maximum-likelihood phylogenetic analysis implemented in the software package PUZZLE 4.0.2 to generate a neighbor-joining tree based on amino acid data [27]. Quartets are bifurcating trees based on subsets of four individuals that can be combined into an overall tree when the set of all individuals is combined. Each puzzling step is analogous to a bootstrap replicate. The program TREEVIEW 1.5.2 was used to display and print neighbor-joining trees [28].

Data analysis.

Threshold values of fluorescence (C_t) obtained for real time reactions were analyzed for differences between experimental organisms and control organisms by determining ratios of experimental to control C_t values using the following formula: target expression relative to control = (efficiency of amplification of target PCR product) $^{(\text{control average } C_t - \text{sample} C_t)}$. Absolute copy numbers of hopper transcripts and genomic copies were determined by comparison to C_t values of a standard curve of known quantities of cloned hopper DNA. Expression ratios between samples were normalized to 18S rRNA as a measure of total RNA of samples relative to controls. Absolute numbers of hopper genomic copies were normalized between samples using relative amounts of SOD2. Relative expression values were identified as outliers and excluded from analysis if they were in excess of one standard deviation from the mean. Students t-test was used to assess the significance of observed differences as implemented by Excel (Microsoft). Correlations of genetic data were performed using GraphPad Prism 3.0 (GraphPad Software Inc.). Statistical analysis of chemistry, survival and growth data was conducted using Sigma Stat version 2.03 (Jandel Scientific). Statistical analysis was conducted using nominal chemical concentration values due to difficulties associated with measuring explosives in sediment. Significant differences in survival and growth of organisms were detected using one-way analysis of variance (ANOVA) and comparisons between treatments were determined using Bonferroni's t-test. Where results did not have a normal distribution, ANOVA on ranks was used followed by Dunn's method for multiple comparisons. For the purposes of these experiments a value of $p < 0.05$ was used for testing of significance for all statistical tests.

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Competing interests statement

The authors declare that they have no competing financial interests.

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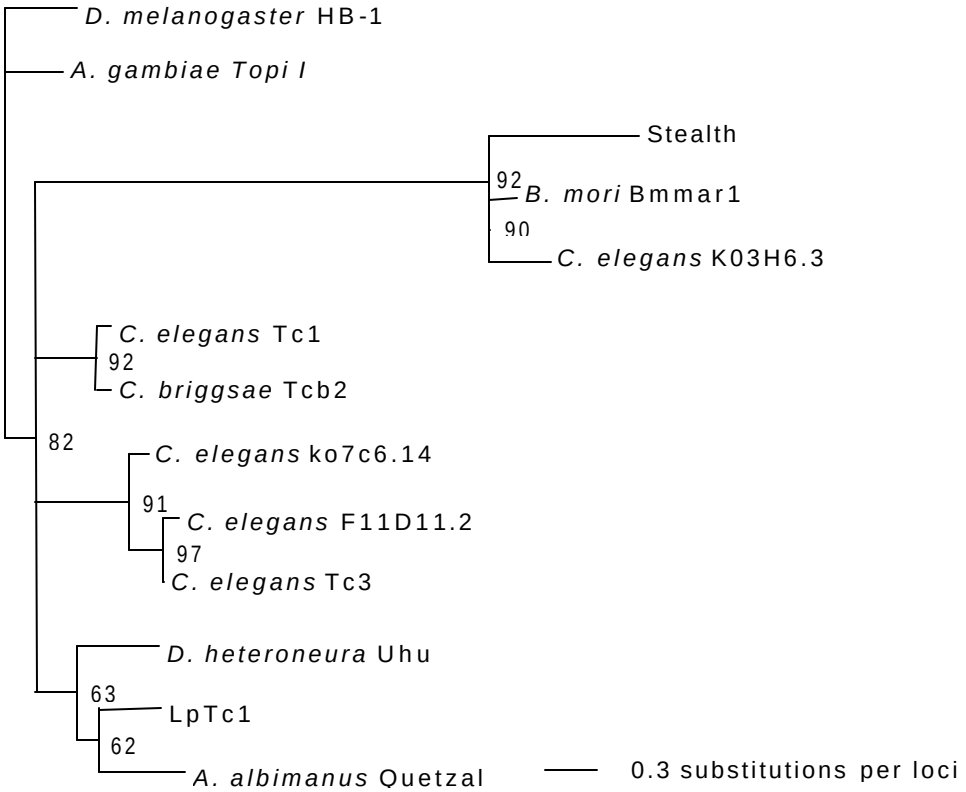
Tables and figures:

Table 1. Putative retrotransposons and transposons cloned from *L. plumulosus*

Clone	Clone [a]	Super family [b]	Similarity [c]	Domain [d]
P450 6-3L5	Ranger	LTR-retrotransposon, gypsy/Ty-3	*Arabidopsis thaliana* Putative retroelement reverse transcriptase similar to gypsy/Ty-3 retroelement (P = 1.2e-16)	rvt , E = 1.8e-12
P450 1-1L2	Dredger	Non LTR-like retrotransposon, Jockey	*Drosophila melanogaster* retrotransposon Jockey reverse transcriptase (P = 2.7e-11)	rvt, E = 6.1e-15
Actin 1-1L4	Hopper		*D. simulans* reverse transcriptase (P = 3.2e-11)	rvt, E = 1.5e-07
Actin 1-1L5	Hopper.1		*D. simulans* reverse transcriptase (P = 1.4e-12)	rvt, E = 8.7e-15
Actin 1-2L3	Hopper.2		*D. simulans* reverse transcriptase (Sum P(2) = 6.0e-06)	rvt, E = 3.0 e-4
Actin 1-3L4	Hopper.3		*D. simulans* reverse transcriptase (P = 4.2e-08)	rvt, E = 3.1e-06
SOD 2-3L1	LpRt1		*Lymantria dispar* gypsy moth LDT1 non-LTR retrotransposon putative endonuclease/reverse transcriptase (Sum P(2) = 2.1e-4)	
SOD 2-4L5	LpRt1.1		*Chironomus tentans* NRLCt2 non-LTR reverse transcriptase from (P = 5.4e-08)	rvt, E = 2.4e-4
Topo 4-1L3	LpRt	Non LTR-like retrotransposon, RTE	*Bos taurus* retrotransposon reverse transcriptase (Score = 169 (59.5 bits), P = 2.9e-11) sequence	
P450 1-1L4	LpRt2	Non LTR-like retrotransposon, CR1	*Schistosoma mansoni* SR1 non-LTR retrotransposon reverse transcriptase (P = 9.1e-15)	rvt, E = 3.8e-12
P450 1-3L4	LpRT3		*S. mansoni* SR2 reverse transcriptase [synthetic construct] (Sum P(2) = 7.2e-18)	
Cyclin 1-1L2	LpRT4		*S. mansoni* SR2 reverse transcriptase [synthetic construct] (Score = 127 (44.7 bits), Sum P(2) = 2.0e-08)	
Cyclin 1-3L2	LpRT5		*Gallus gallus* CR1 (P = 7.5e-10)	rvt, E = 6.6e-4
SOD 2-4L2	Tank	Tc1/mariner transposon	*Caenorhabditis elegans* probable transposase K03H6.3 (P = 6.2e-12)	
P450 1-1L1	Tank.1		*C. elegans* probable transposase K03H6.3 (Sum P(2) = 3.6e-06)	
P450 1-1L5	stealth		*Bombyx mori* Bmmar1 mariner transposon transposase (P = 2.4e-08)	
P450 6-1L1	stealth.1		*B. mori* Bmmar1 mariner transposon transposase (P = 4.0e-08)	
Topo 6-2L5	LpTc1		*Anopheles albimanus* Quetzal: a Tc1 transposon (Sum P(2) = 8.3e-07)	Transposase_5, E = 5.9e-08
Topo 6-2L3	LpTc1.1		*A. albimanus* Quetzal: a Tc1 transposon (Sum P(2) = 1.3e-4)	

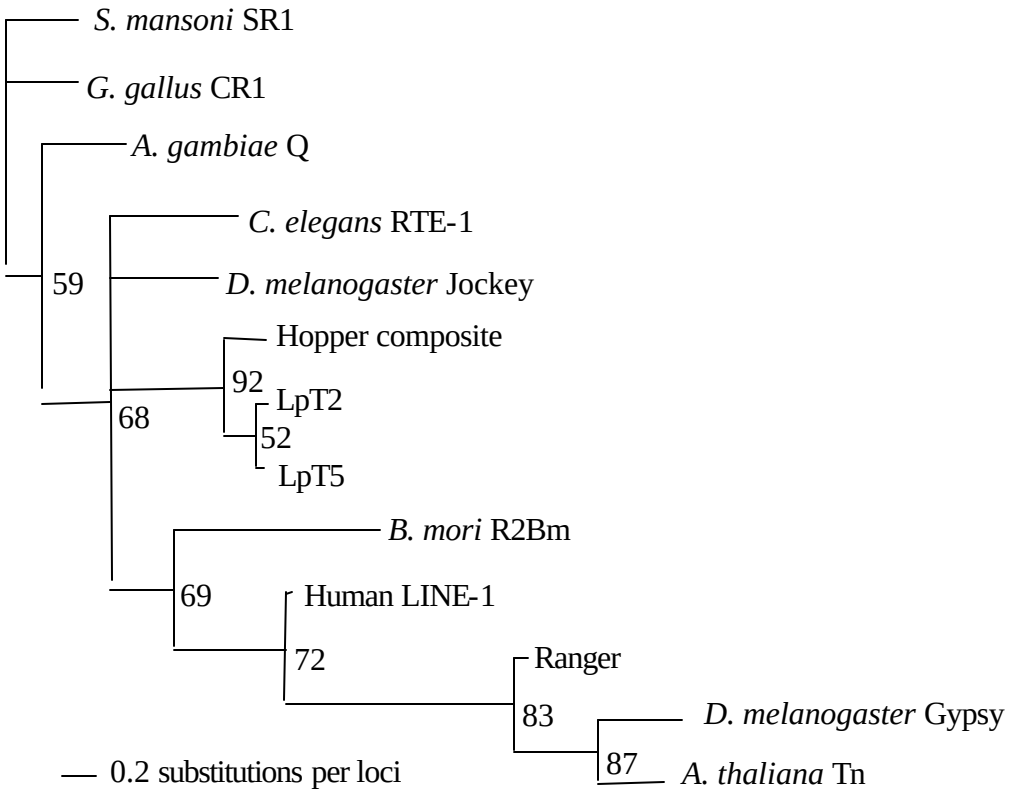
[a] Different clones within a closely related subgroup are represented by identical names followed by a version number (e.g. *hopper* and *hopper.1*). [b] Superfamilies of related retrotransposons elements as described by Malik et al[6]. [c] Highest similarity match of cloned fragment to proteins in the National Center for Biotechnology Information's non-redundant protein database using the programs BLASTX. Sum P(2) indicate sum of probabilities for two noncontiguous open reading froames. [d] Similarity to protein domain family multiple sequence alignments in Pfam 8.0 [24]. rvt= Reverse transcriptase (RNA-dependent DNA polymerase). Transposase_5= Transposase. E-value (expectation) is the number of hits that would be expected to have a score equal or better than this by chance alone.

Figure 1. Comparison of probable transposases of *L. plumulosus* to transposases of Tc1/mariner-like transposons.



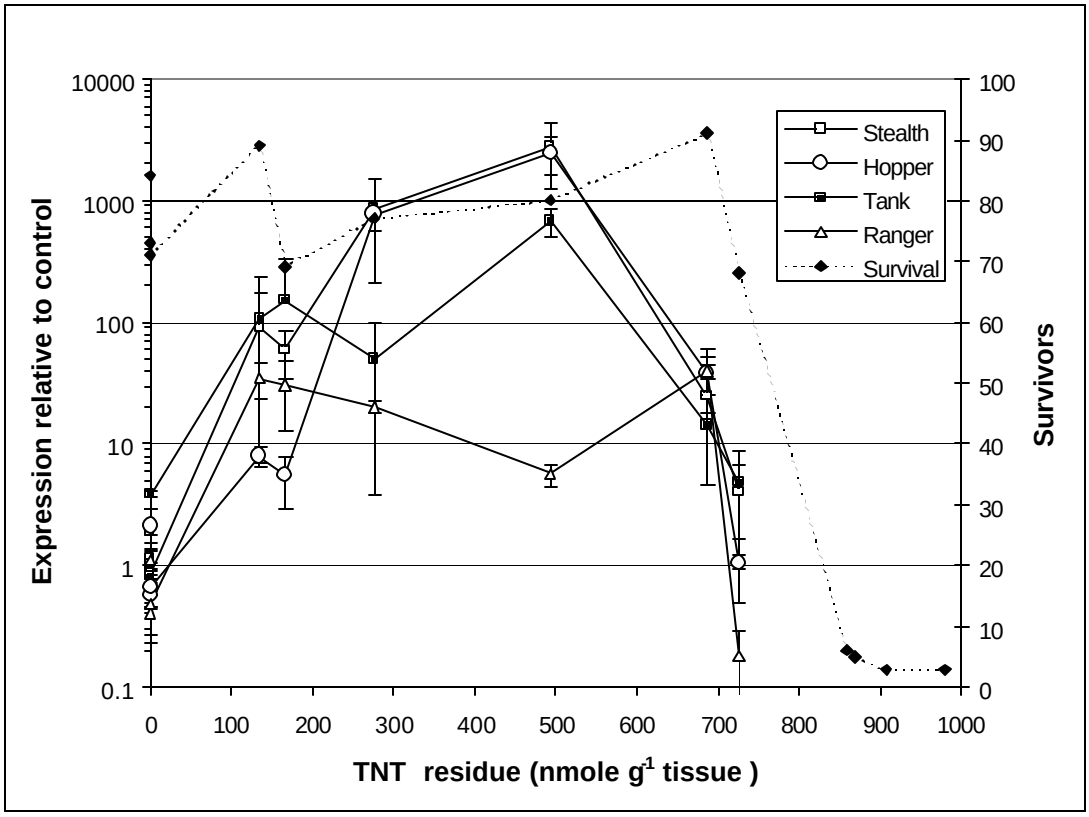
An alignment of the transposase domains of 13 sequences with 92 amino acid sites was used in construction of a quartet puzzling (a majority-rule consensus) neighbor-joining tree with maximum-likelihood branch lengths. One thousand steps were performed using a JTT substitution matrix [29]. Of 715 analyzed quartets, 15.1% were unresolved. Support for the internal branches is shown as percentages of similar branching occurrences in 1,000 iterative puzzling trees. Nodes without values occurred in fewer than 50% of the trees**.** *D.melanogaster* HB-1 = *Drosophila melanogaster* transposon HB-1 (GenBank accession number CAA25884), *A gambiae* Topi I = *Anopheles gambiae* transposon Topi I, Tc1-like transposase (AAD03792), Stealth = *Leptcheirus plumulosus* probable transposon Stealth. *B. mori Bmmar1 = Bombyx mori* mariner transposon Bmmar1 (AAB47739), *C. elegans* K03H6.3= *Caenorhabditis elegans* probable transposase K03H6.3 (T33011), *C. elegans* Tc1= *C. elegans* Tc1 transposase (P03934), *C. briggsae* TC2 = C. briggsae transposase Tcb2 (AAA28149), *C. elegans* ko7c6.14 = *C. elegans* ko7c6.14 hypothetical protein with similarity to transposase (AAB94256), *C. elegans* F11D11.2 = *C. elegans* F11D11.2 hypothetical protein with similarity to transposase (CAB04094), *C. elegans* Tc3 = *C. elegans* transposable element Tc3 transposase (P34257), *D. heteroneura* Uhu = *D. heteroneura* Uhu element transposase (CAA44763), LpTc1 = *L. plumulosus* probable transposon LpTc1, *A. albimanus* Quetzal = *Anopheles albimanus* Quetzal transposase (AAB02109). The clones *tank* and *tank.1* did not have sufficient overlap with the aligned transposase region therefore were not included in tree reconstruction.

Figure 2. Comparison of probable reverse transcriptases of *L. plumulosus* to reverse transcriptases of LTR and non-LTR retrotransposons.



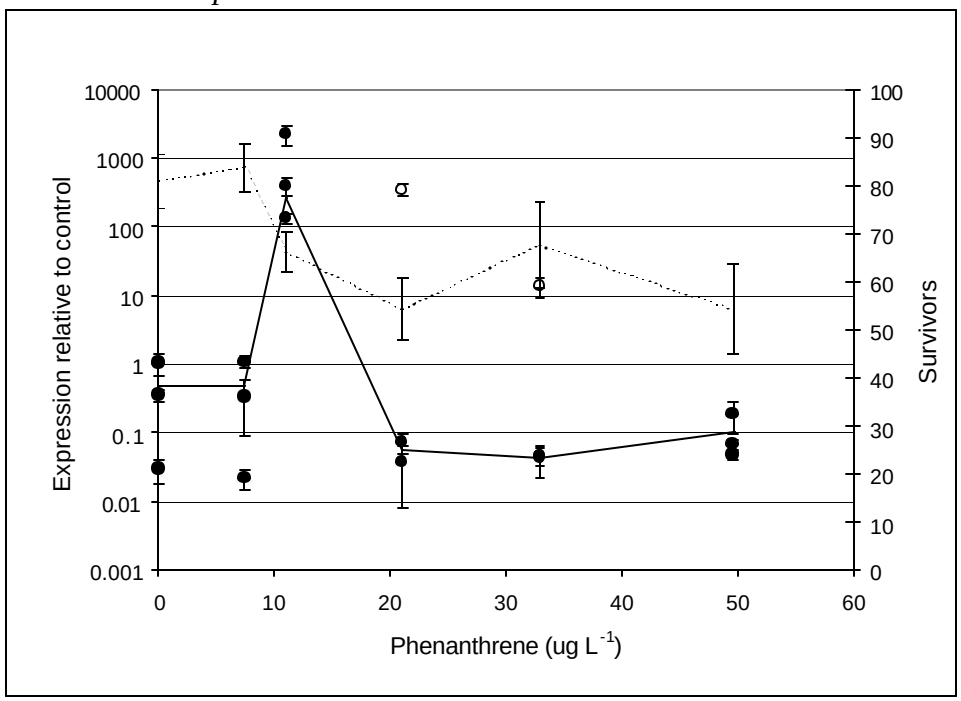
An alignment of reverse transcriptase domains from 13 sequences with 97 amino acid sites was used in construction of a quartet puzzling (a majority-rule consensus) neighbor-joining tree with maximum-likelihood branch lengths. One thousand steps were performed using a blosum 62 substitution matrix [30]. Of 715 analyzed quartets, 13.1% were unresolved. Support for the internal branches is shown as percentages of similar branching occurrences in 1,000 iterative puzzling trees. Nodes without values occurred in fewer than 50% of the trees. *S. mansoni* SR1= *Schistosoma mansoni* SR1 non-Long Terminal Repeat (LTR) retrotransposon (GenBank accession number AAC06263), *G. gallus* CR1 = *Gallus gallus* CR1 non-LTR retrotransposon (AAC60281), *A. gambiae* Q = *Anopheles gambiae* Q retrotransposon (T43020), *C. elegans* RTE-1 = *C. elegans* RTE-1 non-LTR retrotransposon (AAK39312), *D. melanogaster* Jockey = *D. melanogaster* Jockey element (AAA28675), Hopper = *L. plumulosus* probable retrotransposon *hopper* composite, LpRt2 = *L. plumulosus* probable retrotransposon LpRt2, LpRt5 = *L. plumulosus* probable retrotransposon LpRt5, *B. mori* R2BM = *B. mori* rDNA insertion element R2 (AAB59214), Human LIN1 = human LINE-1 reverse transcriptase homologue (P08547), Ranger= *L. plumulosus* probable retrotransposon *ranger*, *D. melonogaster* Gypsy = *D. melonogaster* retrotransposon gypsy POL polyprotein (P10401), *A. thaliana* Tn = *Arabidopsis thaliana* putative retroelement POL polyprotein (AAC69377). The clones *dredger*, LpRt1, LpRt1.1, LpRt, LpRt2, LpRt3, LpRt4, and LpRt5 did not have sufficient overlap with aligned reverse transcriptase domain therefore were not included in tree reconstruction.

Figure 3. *Ranger* and *hopper* reverse transcriptase and stealth and tank transposase transcripts accumulate as a function of TNT tissue residue and survival in L. plumulosus.



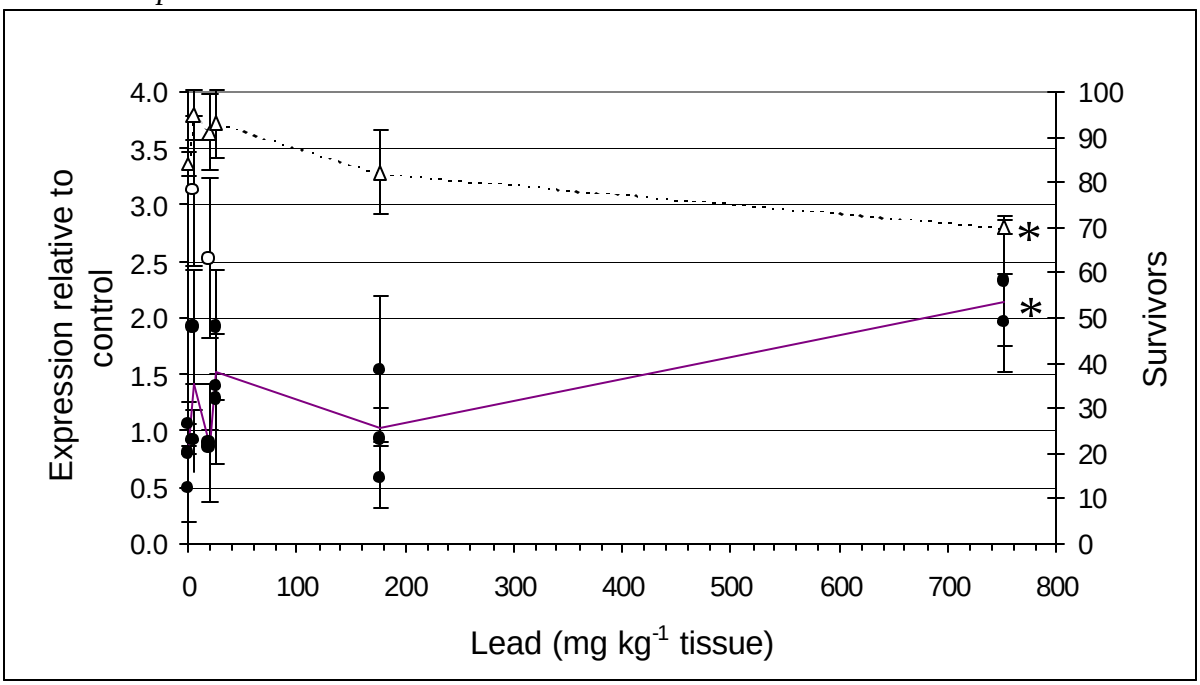
TNT residue represents the total amount of radiolabeled compounds measured in tissues. Expression was normalized to 18S rRNA copies and is relative to that of unexposed controls. Error bars indicate real-time PCR assay replicates for each exposure replicate. The dashed line represents the number of survivors after four days exposure out of 100 individuals

Figure 4. *Hopper* reverse transcriptase transcripts accumulate as a function of phenanthrene exposure and survival in *L. plumulosus*.



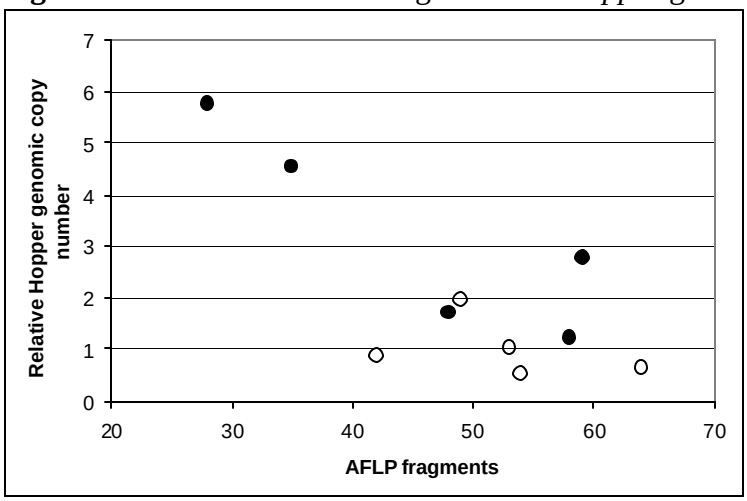
Solid circles represent each exposure replicates of *hopper* reverse transcriptase expression. Open circles are *hopper* replicate outliers excluded from analysis. Expression was normalized to 18S rRNA copies and is relative to that of unexposed controls. Error bars indicate real-time PCR assay replicates of *hopper* expression levels for each exposure replicate. The dotted line represents the number of survivors after four days exposure out of 100 individuals.

Figure 5. *Hopper* reverse transcriptase transcripts accumulate as a function of lead accumulation and survival in *L. plumulosus*.



The solid line indicates means values for exposure replicates. Solid circles represent each exposure replicates of *hopper* reverse transcriptase expression. Open circles are Hopper replicate outliers excluded from analysis. Triangles represent actin exposure replicate values. Expression was normalized to 18S rRNA copies and is relative to that of unexposed controls. Error bars indicate real-time PCR assay replicates for each exposure replicate. The dotted line represents the number of survivors after four days exposure out of 100 individuals. Asterisks indicate means significantly different from controls.

Figure 6. Correlation of AFLP fragments with *hopper* genomic copy number.



Hopper copy number and AFLP analysis were determined from replicates consisting of populations of 20-pooled individuals. Copy number values are relative to the mean value of controls. Solid circles represent populations exposed to 5 mg L^{-1} TNT and open circles control populations.

Appendix A2: Technical reports:

None

Appendix A3: Conference/Symposium Proceedings Papers (other than Abstracts) that are scientifically recognized and referenceable:

None

Appendix A4: Published Technical Abstracts

Diedrich, M., E. Perkins, T. Bridges, and H. Fredrickson. Nov. 2000. Transcriptional-level responses of sedimentary macrofauna to explosives. Society of Environmental Toxicology and Chemistry 21st Annual meeting. Nashville, TN. November, 2000.

Rapid methods for generating meaningful sub-lethal toxicity data on sediments containing moderate to low levels of explosives are needed to discriminate between sites requiring active remediation from those where active remediation would be more detrimental than passive remediation. Two marine (*Neanthes arenaceodentata, Leptocheirus plumulosus*) and 2 freshwater (*Hyalella azeteca and Chironomus tentans*) macrofaunal species were chronically exposed to TNT in a dose-response format. After exposure, whole-organism end points (mortality, growth, and reproductive success) were compared to gene expression levels in the organisms. High-density DNA arrays were developed to monitor stress response and novel, explosives responsive gene expression levels. Microarrays were created using cloned gene fragments from two different sources- conserved genes and novel genes identified by differential expression in response to TNT. Fragments of known stress response genes in the test species were isolated using consensus-degenerate primers. 100 to 200 gene fragments were cloned from each organism and identified by sequencing. Gene fragments were identified that were similar to actin, superoxide dismutase, DNA topoisomerase I, reverse transcriptase, and protein kinases. Novel genes, whose expression was effected by TNT exposures, were isolated using differential display techniques (arbitrarily primed differential display and restriction enzyme differential display). Expression of a putative p450 gene was found to increase over 80-fold in response to TNT exposure before any whole organism effects were observed.

Diedrich, K., E. Perkins, and H. Fredrickson. Nov. 2000. DEODAS: DEgenerate Oligonucleotide Design and Analysis System for design of DNA microarrays for environmental assessment applications. Society of Environmental Toxicology and Chemistry 21st Annual meeting. Nashville, TN. November, 2000.

High-density DNA arrays enable the simultaneous detection of thousands of genes and measurement of their expression levels (e.g., toxicogenomics). DNA arrays for assessing stress and damage caused by pollutants to microorganisms have wide ranging applications in toxicology, site assessment and bioremediation efforts. However, up to 99% of microorganisms

in the environment are uncharacterized. Application of toxicogenomic strategies to native organisms in the environment requires detection of genes whose exact sequences are often not known. Consensus probes based on conserved protein sequences have been successfully used to detect genes in related families. Translation of a single protein sequence requires multiple nucleic acid sequences to compensate for degeneracy of the nucleic acid code. Until now, rational design of consensus-degenerate oligonucleotide probes and PCR primer pairs required the use of multiple programs running on different computer platforms and required extensive manual reformatting of data between applications. This was feasible when one protein of interest was targeted, but this process rapidly becomes unwieldy when designing 10 - 1000 probes for high-density arrays. A GNU/Linux, PC-based system (DEODAS) for designing and electronically analyzing consensus-degenerate probes was developed based on published software tools: ClustalW, CODEHOP, and EMBOSS. DEODAS integrates these software tools together to automatically design and screen probes in a batch format against existing databases of gene sequences. This greatly decreases the amount of interactive time required to design and screen probes. Output on designed probes is organized in a searchable database that is used to examine the oligonucleotide data. The utility of DEODAS is demonstrated by the design of oligonucleotide probes targeting genes encoding enzymes involved in DNA repair, metabolism of xenobiotics, and general stress response in bacteria.

Duke, B.M., Lotufo, G.R., Bridges, T.S. and J.A. Steevens. Toxicological evaluation and comparison of explosives using freshwater macroinvertebrates. Society of Environmental Toxicology and Chemistry 21st Annual meeting. Nashville, TN. November, 2000.

Explosives occur as contaminants in soil, water, and sediment. The toxicological effects of *2,4,6-Trinitrotoluen*e (TNT), 1,3,5-trinitrobenzene (TNB), 2,4-diamino-6-nitrotoluene (2, 4-DANT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) were evaluated in sediment exposures. These compounds were selected for study based on military relevance, chemical class and toxicological mechanism. Model organisms utilized for the study included *Hyalella azteca* and *Chironomous tentans*, with survival and growth as toxicological endpoints. Experiments were conducted following U.S. EPA methods for sediment toxicity tests utilizing spiked sediments. Survival of adult *H. azteca* was significantly decreased following exposure to 400 mg/kg TNT and as low as 100 and 400 mg/kg DANT. However, TNB did not result in statistically significant effects on survival at concentrations as high as 400 mg/kg. Juvenile *H. azteca* survival was significantly reduced at concentrations greater than 50 mg/kg TNT, 100 mg/kg TNB, and 50 mg/kg DANT. RDX and HMX did not elicit significant toxicological effects at concentrations as high as 250 and 200 mg/kg, respectively. In addition, statistically significant effects were observed on the growth of *H. azteca*. Midge survival was significantly decreased compared to the control at concentrations of 200 mg/kg TNT, 400 mg/kg TNB and 400mg/kg DANT. There were no significant effects on survival when using RDX and HMX at concentrations up to 1000 mg/kg or 200 mg/kg, respectively. RDX and HMX resulted in a significant increase in growth, potentially through a hormetic mechanism. Explosives have similar chemical structures and would be expected to act with similar potencies and mechanisms of action. However, results of

the current study demonstrate substantial variability in responses of macroinvertebrates following exposure to explosives.

Houston, J.G., Duke, B.M. and J.A. Steevens. Examination of the toxicity of TNT and degradation products in three aquatic invertebrates. Society of Environmental Toxicology and Chemistry 21st Annual meeting. Nashville, TN. November, 2000.

Comparative toxicity of 2,4,6-Trinitrotoluene (TNT) and three of it's degradation products, 2,4-Diamino-6-nitrotoluene (DANT), 2-amino-4,6-dinitrotoluene (2-ADNT), and 1,3,5-trinitrobenzene (TNB) was determined utilizing three aquatic invertebrates. Model organisms in the study were *Neanthes areanceodentata*, a marine polychaete, *Leptocheirus plumlosus*, an estuarine amphipod, and *Hyalella azteca*, a freshwater amphipod. Water-only experiments were conducted to develop concentration response curves with survival as the toxicological endpoint. Concentration response curves were used to assess the slope and determine EC_{50} values for each compound. Additional comparisons of the effects of the toxicants were conducted through graphical analysis. In the current study survival effects were observed for *N. arenaceodentata* with TNT and 2-ADNT (EC_{50} = 29.3 and 36.8 µM respectively) , but not for DANT (no mortality up to 491 µM). To further investigate the differences in toxicity among compounds, pretreatment of organisms with nitroreductase inhibitors was used to block their metabolism and degradation. Experiments were conducted to establish a greater understanding of the mechanistic action of TNT toxicity. These results demonstrate that the toxicity of TNT may be elicited through the formation of azoxy and nitroso degradation products.

Lotufo, G.R., Steevens, J.A., Larson, S.L. and T.S. Bridges. Can we derive critical body residues for nitroaromatic compounds? Society of Environmental Toxicology and Chemistry 21st Annual meeting. Nashville, TN. November, 2000.

Nitroaromatic explosive compounds were extensively released to the environment at military sites in the U.S. and throughout the world over the last 100 years, resulting in high levels of contamination in surface water, ground water, soils, and sediments. We evaluated the toxicokinetics of trinitrotoluene (TNT) to the estuarine amphipod *Leptocheirus plumulosus*. Water exposures to TNT resulted in decreased survival (96-h LC50 = 2 mg/L). Apparent steady state tissue concentration was attained in less than 48 h. Following a 2-day exposure period, the total fraction of radiolabel attributable to parent TNT and its known degradation products corresponded to only 6% of the total radioactivity in the tissues. Liquid/liquid extraction and gel permeation chromatography indicated that most of the radiolabel that was originally TNT became bound to organic molecules forming complexes that were more polar and larger than the parent molecule. Therefore, close to all the TNT taken up from the water was biotransformed and conjugated to organic molecules and resisted solvent extraction. Sediment exposures to 2,4-diaminonitrotoluene and trinitrobenzene also resulted in decreased survival of *Leptocheirus*. However, the concentration of solvent extractable parent compound or potential metabolites in the tissues were below the method detection limits (0.1 µg/g). These results indicated that TNT and other nitroaromatic compounds commonly found in the

environment may elicit their toxicity through bioactivation to more reactive and toxic metabolites. Critical body residues (CBRs) for TNT reported in the literature (0.04-0.22 mmol/kg) refer to the TNT-molar-equivalents concentration of radiolabeled compounds in the tissues and were not confirmed by chemical analysis. Understanding of the chemical structure of nitroaromatic tissue residues and the nature of their binding to macromolecules will be necessary before CBRs can be derived for these compounds.

Perkins. E., H. Fredrickson, T. Bridges, J. Fleming, K. Beattie, R. Tonucci. 2000. Development of High-Density DNA Arrays for Ecological Risk Assessment. Society of Environmental Toxicology and Chemistry 21st Annual meeting. Nashville, TN. November, 2000.

Assays based on DNA probes can be used to monitor effects of environmental contaminants on biological systems. Recent engineering advances have enabled high-density spotting of hybridization probe arrays (thousands per cm^2), automated hybridization, data capture and analysis. We are developing flow-through, high-density DNA hybridization arrays targeting genetic markers for rapid screening of environmental toxicity during site characterizations and remediation efforts. Two types of arrays are being developed. The first focuses upon effects of toxicants on bacterial populations resident in soils and sediments. The second focuses upon rapid assessment of exposure and toxicity in macrofauna used in chronic, sub-lethal toxicity tests. To support assay development, a 28-sample flow-through chip device was created to increase sample throughput. A comprehensive suite of computer programs has been made to enable design of large numbers of oligonucleotides for hybridization on microarrays. Specific genes for use in a bacterial toxicity microarray were defined by examining responses of *Escherichia coli* to Uranium and trinitrotoluene exposures. Whole genome arrays (Panorama membranes, Sigma-Genosys) identified several genes affected by chemical exposure. These genes are being examined to create smaller arrays for use in the multi-sample format. Macrofaunal arrays are being designed by isolation of conserved genes involved in stress from target organisms in addition to identification of new genes as markers of exposure.

Perkins, E.J., Houston, J.G., Lotufo, G.R., Steevens, J.A., Bridges,T.S. and Fredrickson, H.F. 2001. Gene Expression Profiling of 2,4,6-trinitrotoluene Toxicity in *Chironomus tentans*. Society for Environmental Toxicology and Chemistry annual meeting, Baltimore, MD.

The Department of Defense is responsible for clean up of many sites contaminated with explosives such as 2,4,6-trinitrotoluene (TNT). The cost of site remediation can be prohibitively expensive, limiting its application. To permit more efficient remediation projects with realistic cleanup goals, bioassays for toxicity to identify relevant biological endpoints for cleanup goals. These assays are generally based upon whole organism effects such as morbidity, growth and fecundity and require 10 to 30 days to complete. We are investigating the effects of TNT exposure in a freshwater invertebrate Chironomus tentans, to better understand toxicological mechanisms and develop rapid screening methods to assess contaminant exposure in test organisms. Tissue was collected from 2nd instar organisms following exposure to four concentrations of TNT and at 1 hour, 1, 4, and 10-day time points. Concentrations for the

exposure were selected at the EC50 (2 mg/L), LOEC (1 mg/L), and two concentrations below the LOEC (0.1, and 0.5 mg/L). Exposures were conducted to ensure that organisms were the same size and age at the time of tissue harvest. Replicates were examined for gross level end points (survival, growth and total RNA per ug tissue) and were examined for differential expression of stress related genes using a panel of primers targeting conserved genes as well as restriction fragment differential display. Abnormally high total RNA levels correlated with growth LOEC at 10 days exposure. In addition, several stress related genes were found to change expression with exposure.

Lotufo, G.R., Farrar, J.D., Steevens, J.S. 2001. Mixture Toxicity of TNT and TNT Breakdown Products to the Freshwater Midge *Chironomus tentans*. Society for Environmental Toxicology and Chemistry annual meeting, Baltimore, MD.

The toxicities of trinitrotoluene (TNT) and its breakdown products trinitrobenzene (TNB), 2-aminodinitrotolune (ADNT) and 2,4-diaminonitrotoluene (DANT) were determined using third instar *Chironomus tentans*, in 10-day aqueous and spiked-sediment exposures. The magnitude of the lethal effects of TNT, TNB and ADNT was similar, whereas DANT was substantially less potent (nominal 10-d LC50 5 9, 9, 20, and 235 mmol/L for TNT, ADNT, TNB, and DANT, respectively). In sediment, TNT and ADNT were more potent than TNB and DANT (nominal 10d LC50 5 280, 259, and 1049 mmol/kg for TNT, ADNT, TNB, and DANT, respectively). Mixture experiments were performed to investigate the nature of the toxicological interactions of nitroaromatic compounds. The molar LC50 for mixtures of TNT, TNB, ADNT and DANT was 17 mmol/L for water and 530 mmol/kg for sediment. The relative contribution of each compound to the overall toxicity of the mixture was estimated for each treatment using toxic units (compound concentration divided by compound LC50). The sum toxic units for each treatment and the survival data were used in the calculation of an experimental sum toxic unit LC50 for the mixture. The sum toxic unit LC50, calculated using nominal concentrations, was 1.04 for the water experiment and 1.05 for the sediment experiment, as expected when compounds interact additively to promote biological effects. Information on the relative toxicity of TNT and its major breakdown products and their toxicological interactions in mixture exposures is valuable for assessing ecological risk of contaminated sites.

Steevens, J.A. and Gibson, A.B. 2001. Toxicokinetic Interactions of Trinitrotoluene with Lead and Phenanthrene. Society for Environmental Toxicology and Chemistry annual meeting, Baltimore, MD.

The toxicokinetic interactions of 2,4,6-trinitrotoluene (TNT) in combination with metals and polycyclic aromatic hydrocarbons (PAHs) were assessed utilizing an aquatic invertebrate, *Hyalella azteca*. Lead and phenanthrene were selected as model toxicants. The toxicokinetic interactions of binary mixtures of TNT, lead, and phenanthrene were characterized through a one-compartment kinetic model. Initially, the uptake and elimination kinetics of TNT were determined. Radiolabeled C14-TNT and C14-phenanthrene were used to evaluate uptake and accumulation of the organic compounds. The uptake coefficient (Ku) during a 4-day exposure to TNT was 2.41 dpm/mg. Elimination, as indicated by the elimination constant (Ke), was (-

0.0228 dpm/mg) following transfer to toxicant-free water. Effects of simultaneous and sequential binary mixture exposures on the uptake and elimination of all three chemicals was evaluated. Sequential exposures were conducted with a 2-day pre-exposure to the second chemical prior to exposure to the chemical evaluated for bioaccumulation. Toxicokinetic interactions were observed for the TNT and phenanthrene mixture. While no significant differences were observed for the rate of uptake (Ku range 0.034 - 0.062) of phenanthrene in the presence of TNT, the Ke of phenanthrene increased significantly following simultaneous and sequential treatment with 0.2 mg/L TNT. Toxicokinetic interactions of TNT, phenanthrene, and lead can directly alter the tissue concentration following exposure, and may potentially modify the resulting toxicity of these compounds.

Perkins, E. J. 2002. Genetic tools for assessing exposure to contaminants. Abstract for Mississippi Water Resource Conference, Raymond, Mississippi.

The Department of Defense is responsible for sites contaminated with chemicals such as 2,4,6-trinitrotoluene (TNT) and polyaromatic hydrocarbons (PAHs). To permit more efficient remediation projects with realistic cleanup goals, bioassays for toxicity are used to identify relevant biological endpoints for cleanup goals. These assays are generally based upon whole organism effects such as morbidity, growth and fecundity, require 10 to 30 days to complete. We are investigating the effects of contaminant exposure to develop rapid screening methods for toxicity assessment in test organisms such as *Chironomus tentans* and *Leptochierus plumulosus*. We have isolated a number of genes that are responsive to TNT and a model PAH, fluoranthene. Semi-quantitative real time reverse transcriptase polymerase chain reaction assays were developed to monitor gene expression in both organisms. Gene expression levels responded in a dose dependent manner to TNT and fluoranthene. Expression levels were highly correlated with lethal and sublethal endpoints. In addition to known chemicals, water and sediment contain uncharacterized compounds that are a source of toxicity. We are investigating the use of gene expression fingerprints to identify compounds to which an organism is exposed. Early evidence indicates that such fingerprints are characteristic of the compounds to which the organism was exposed. Each of these approaches hold great promise in improving toxicity assessment.

Lotufo, G. R., Farrar J. D., Steevens J. A., Bridges, T.S. 2002. Toxicity and bioaccumulation of TNT and TNT breakdown products in the midge Chironomus tentans in water and sediment exposures. Abstract for SETAC Europe 12th Annual Meeting, Vienna, Austria.

Nitroaromatic explosive compounds were extensively released to the environment at military sites in the U.S. and throughout the world over the last 100 years, resulting in high levels of contamination in surface water, ground water, soils, and sediments. The toxicity of trinitrotoluene (TNT) and its breakdown products trinitrobenzene (TNB), 2- aminodinitrotolune (ADNT) and 2,4-diaminonitrotoluene (DANT) were determined using the freshwater midge Chironomus tentans. In water exposures, the magnitude of the lethal effects of TNT, TNB and ADNT was similar, whereas DANT was substantially less potent. In sediment, transformation and resistance to solvent-extraction following spiking were observed for all compounds, most

extensively for TNT and TNB. TNT and ADNT were more potent than TNB and 2,4-DANT. Toxic effects on growth were observed in water but not in sediment exposures. Mixture experiments were performed to investigate the nature of the toxicological interactions of nitroaromatic compounds. The sum toxic unit LC50 approximated 1 in water and sediment experiments, as expected when compounds interact additively. In water exposures, the lethal body residue (LBR) for all compounds ranged from 0.01 to 0.03 mmol/kg wet wt. for all compounds, except for TNB, which was not detected in tissue extracts. The fate of TNT in tissues and its LBR were examined in 4-day water exposures to radiolabeled TNT. A mass balance of the radioactivity in the tissue revealed that only 60% of the radioactivity was removed by acetonitrile extraction and the total concentration of nitroaromatics measured using HPLC, mostly TNT breakdown products, corresponded to only 7% of the concentration of TNT molar- equivalents. Therefore, most of the TNT taken up from the water was biotransformed to compounds that either were undetected by HPLC or conjugated to organic molecules and resisted solvent extraction. The LBR was 1.25 mmol/kg for TNT molar-equivalents but much lower, 0.04 mmol/kg, for sum measured nitroaromatics. The toxicological significance of the unidentified/unextractable fraction remains unknown. Research aiming to characterize the biochemical fate of nitroaromatic compounds in invertebrate tissues is being conducted to allow a better understanding of the mechanisms of toxic action of these highly reactive compounds.

Perkins E, Lotufo G, Farrar D. 2002. Correlation of lethal and sublethal effects of 2,4,6-trinitrotoluene (TNT), phenanthrene, and lead with gene expression in the marine benthic amphipod *Leptochierus plumulosus*. Society for Environmental Toxicology and Chemistry Annual Meeting, Salt Lake city, UT, USA

Thousands of yards of sediment are dredged and disposed of each year. Contaminated sediments must be assessed for the potential to cause harmful effects after disposal. Traditionally, bioaccumulation or toxicity has been measured using difficult analytical tests and gross level endpoints such as growth and mortality. We have employed quantitative gene expression assays to better understand effects of exposure to energetic compounds, polyaromatic hydrocarbons, and metals on the marine benthic amphipod Leptochierus plumulosus at lethal and sublethal exposures and provide alternate measures of bioaccumulation and toxicity. *L. plumulosus* was exposed to a dose series of 2,4,6-trinitrotoluene (TNT), phenanthrene, or lead. Survival and tissue residues of chemicals were measured and compared to expression levels of a panel of ten genes. Assays were used targeting actin, a putative polyprotein, two different superoxide dismutases, two reverse transcriptases, a 60S ribosomal protein, a 26S protease regulatory subunit, a transposase, and a gene of unknown function, b04. Several genes exhibited increased expression at low level exposures of TNT followed by significantly reduced expression approaching toxic levels of TNT. Lead exposures had markedly different effects on gene expression. Increasing lead concentrations had no effect on 26S protease regulatory subunit expression, whereas effects on 60S ribosomal protein and b04 gene expression were only observed at levels of lead that caused significant mortality. Expression levels of superoxide dismutase gene decreased with lead exposure in a dose

dependent manner. Reduction of superoxide dismutase levels would result in a decreased ability to prevent oxidative stress injuries, consistent with observations that pre-exposure to lead can enhance TNT toxicity.

Bridges, T.S., Lotufo, G., Perkins, E. J., Fredrickson, H. L., Farrar, D., Steevens, J. 2002. Aquatic toxicology of explosives: Fate, toxicity, critical body residues, and effects on gene expression. Partners in Environmental Technology Technical Symposium & Workshop, sponsored by SERDP and ESTCP

The explosives TNT, RDX, HMX, and their degradation products are contaminants of concern typically associated with military activities. The toxicity of TNT and the TNT breakdown products 2-aminodinitrotolune (2ADNT), 2,4-diaminonitrotoluene (2,4DANT) and trinitrobenzene (TNB) were compared using fish and benthic invertebrates. In water exposures, 2,4DANT was substantially less toxic than the other compounds for the midge *Chironomus tentans* and the fish *Cyprinodon variegatus*. Radiolabeled TNT spiked to fresh water and marine sediments rapidly breaks down to ADNT and DANT congeners and a substantial portion of the compounds move into the overlying water during static exposures. The non-solvent-extractable fraction of the radiolabel in sediment increases with increasing aging of the sediment. In sediment exposures using *C. tentans*, concentrations associated with toxicity were similar for all compounds. Most of the TNT molecules accumulated by aquatic invertebrates appear to rapidly transform to ADNT or DANT and to strongly bind to organic molecules becoming non-extractable and non-detectable by traditional chemical analysis. Critical body residues determined as the sum molar concentrations of chemically detected nitroaromatics were similar for midges (0.04 mmol/kg) and fish (0.02 mmol/kg). The explosives RDX and HMX did not decrease invertebrate survival in water or sediment exposures even at the water solubility limit or at exceedingly high sediment concentrations (>1,000 mg/kg). To better understand effects at the cellular level, genetic assays were developed for assessing effects of contaminants on benthic invertebrates. These assays were found to correlate with tissue residue concentrations and whole organism survival effects. In the amphipod *Leptocheirus plumulosus*, 10 different genes were examined. Low levels of TNT caused a general increase in gene expression, with expression decreasing prior to lethal effects being observed. Several mobile genetic elements were isolated from *L. plumulosus*. One element, Hopper, was activated by sub-lethal concentrations of TNT and phenanthrene but not lead. Exposure to TNT induced movement of Hopper and may result in unexpected genotoxic and mutational events. Lead exposure effects were observed on superoxide dismutase gene expression that could result in sensitization of *L. plumulosus* to subsequent TNT exposures. Since significant effects were observed at sublethal levels of TNT exposure, genetic assays are useful as tools in screening field-contaminated sediments. Our investigation of the toxicity, mechanisms of action, bioaccumulation, and mixture interactions for TNT will provide valuable information when extrapolating laboratory data to ecological risk assessments. **This research was funded by SERDP (CU-1129).**

Appendix A5: **Published Text Books or Book Chapters**

None.

APPENDIX B:

Table 5.b.1.A. Clones with high similarity to known genes isolated by CODEHOP PCR and differential display.

RT PCR Assay	Clone	Similarity	
	Cyclin 1-1L3	probable pyruvate dehydrogenase E1 component, beta subunit (Score = 185, P = 4.1e-13)	
	SOD 2-4L4	similar to transporter binding protein [Bacillus subtilis] Score = 137 (48.2 bits), Expect = 2.7e-07, P = 2.7e-07 peptide ABC transporter, periplasmic peptide-binding protein - Vibrio cholerae Score = 129 (45.4 bits), Expect = 1.8e-06, P = 1.8e-06	
SOD2	SOD 2-4L3	Mn-superoxide dismutase [Rhodobacter capsulatus] Score = 390 (137.3 bits), Expect = 3.5e-35, P = 3.5e-35	
Actin	Actin 2-1L4-	alpha-actin, muscle; Score = 321 (113.0 bits), Expect = 7.1e-28, P = 7.1e-28 The alpha actins are found in muscle tissues and are a major constituent of the contractile apparatus and cell movement. Changes in expression may result in changes in cell structure and growth.	
26S	A04_C12_02 (26S)	26S PROTEASE REGULATORY SUBUNIT Homo sapiens S10B (P44) (CONSERVED ATPASE DOMAIN PROTEIN 44 Score = 332 (116.9 bits), Expect = 4.9e-29, P = 4.9e-29 26S protease regulatory subunit is part of the major non-lysosomal protease in eukaryotic cells. It is involved in degrading both cytoplasmic and nuclear proteins. Changes in expression may indicate an unbalance in general protein stability.	
B4	B4_C12b_04 (B4)	Unknown	
QM	C04_12c_06 (QM)	QM PROTEIN HOMOLOG [Drosophila melanogaster] Score = 891 (313.6 bits), Expect = 2.7e-91, Sum P(2) = 2.7e-91; QM protein [Bombyx mandarina] Score = 870 (306.3 bits), Expect = 2.7e-89, Sum P(2) = 2.7e-89, Identities = 159/195 (81%), QM PROTEIN HOMOLOG. QM, a novel gene that was firstly isolated as a putative tumor suppressor gene from Wilms' tumor cell line. Although it is well known that the QM gene product plays an important role within the tumor cells, the precise role of QM in the non-tumor cells has remained elusive. QM homologue from Bombyx mandarina exhibits tissue-/stage-dependent expression – may have an important biological role associated with pupae formation.	
tank	A DNA transposon. A mobile genetic element that can create copies of itself and insert throughout the genome. These elements can replicate, move and insert into different chromosomal DNA locations creating a source of genotoxicity and mutagenicity.		
ranger	A retrotransposon. A mobile genetic element that can create copies of itself and insert throughout the genome. These elements can replicate, move and insert into different chromosomal DNA locations creating a source of genotoxicity and mutagenicity.		
stealth	A DNA transposon. A mobile genetic element that can create copies of itself and insert throughout the genome. These elements can replicate, move and insert into different chromosomal DNA locations creating a source of genotoxicity and mutagenicity.		
hopper	A retrotransposon. A mobile genetic element that can create copies of itself and insert throughout the genome. These elements can replicate, move and insert into different chromosomal DNA locations creating a source of genotoxicity and mutagenicity.		

Figure 5.b.1.A. Effect of TNT on (A) mortality and (B) tissue residue in 4-day exposures.

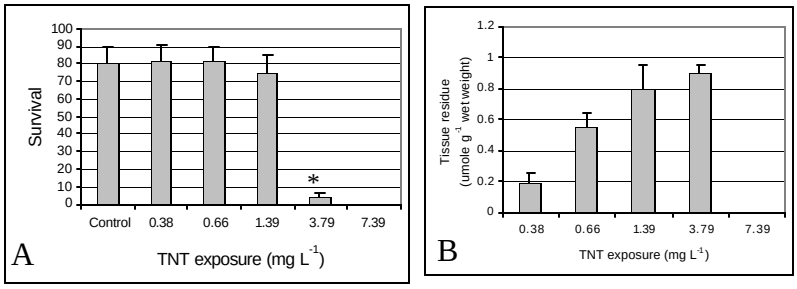
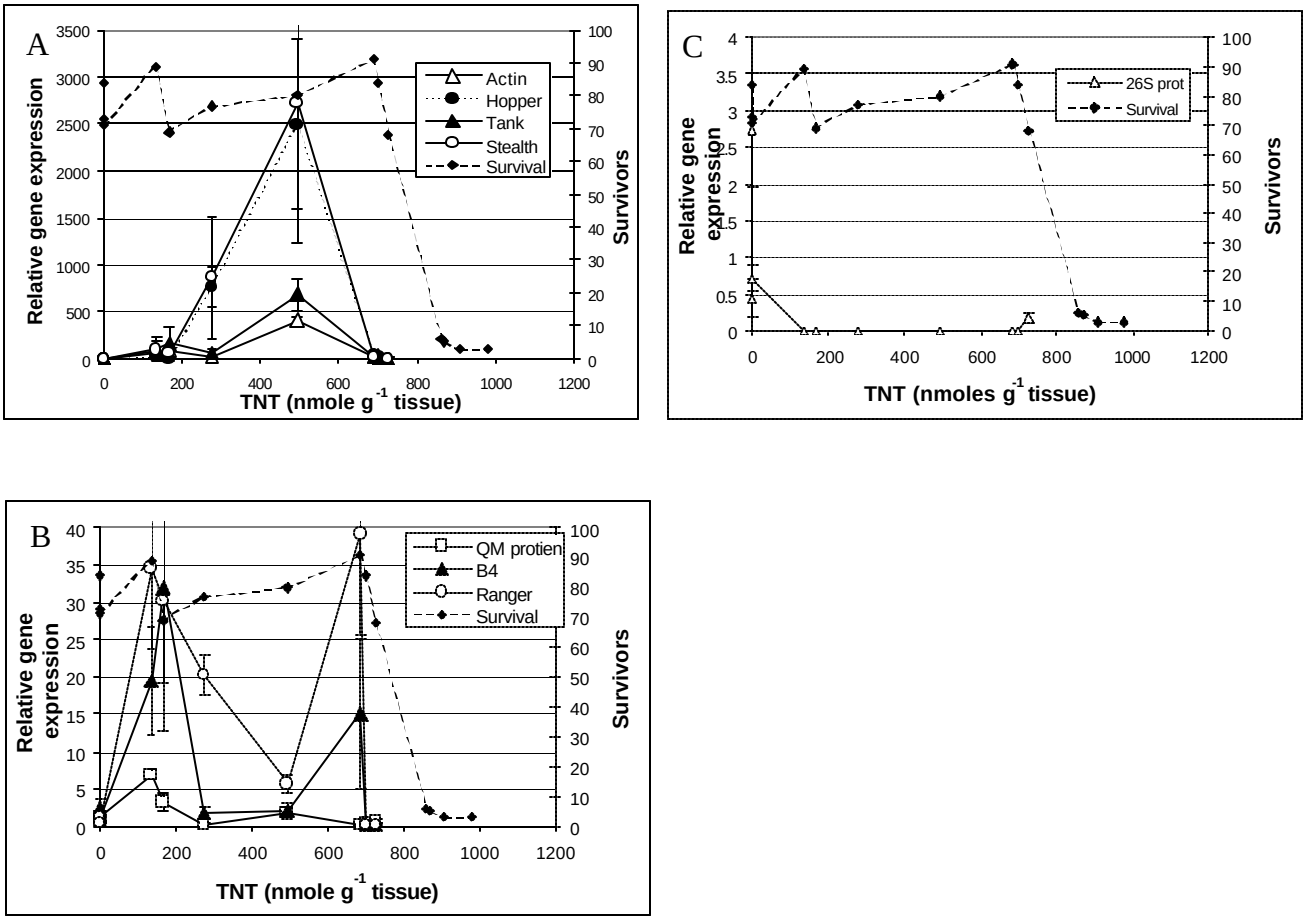
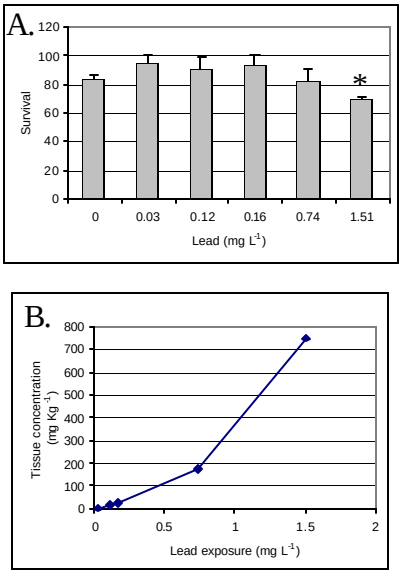


Figure 5.b.1.B. TNT caused a general increase in gene expression, with expression decreasing prior to lethal effects being observed.



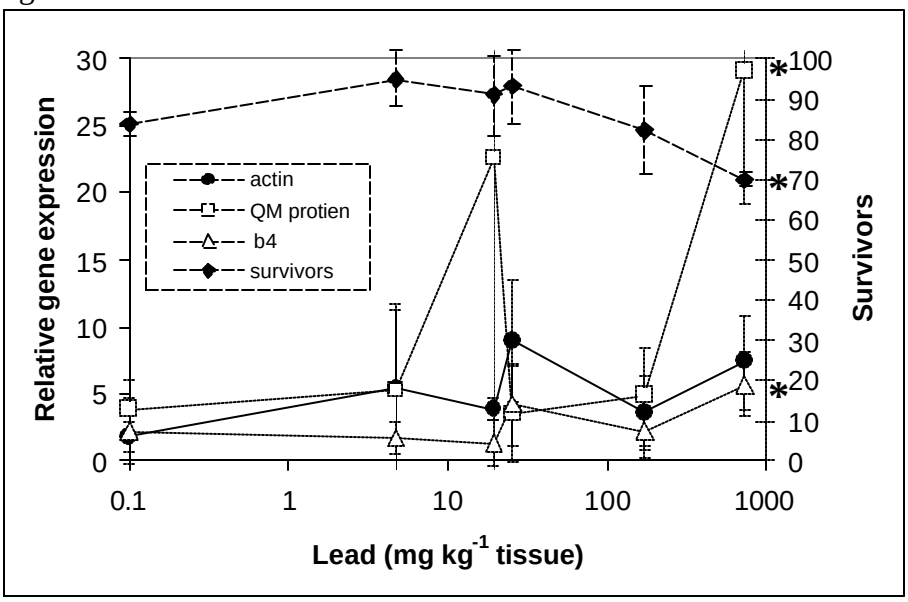
Panel A, genes strongly effected by TNT. Data for DNA transposons *tank* and *stealth*, and the retrotransposon *hopper* have been taken from Perkins et al., (2003, appendix A). Panel B, genes moderately effected by TNT. Data for the retrotransposon *ranger* has been taken from Perkins et al., (2003, appendix A). Panel C, reduction of 26S regulatory protein expression with TNT exposure. Error bars indicate real-time PCR assay replicates for each exposure replicate. Solid diamonds with a dashed line represent the number of survivors of 100 individuals after four days exposure.

Figure 5.b.1.C. Effect of 4-day aqueous lead exposure on (A) survival and (B) tissue concentrations in *L. plumulosus*.



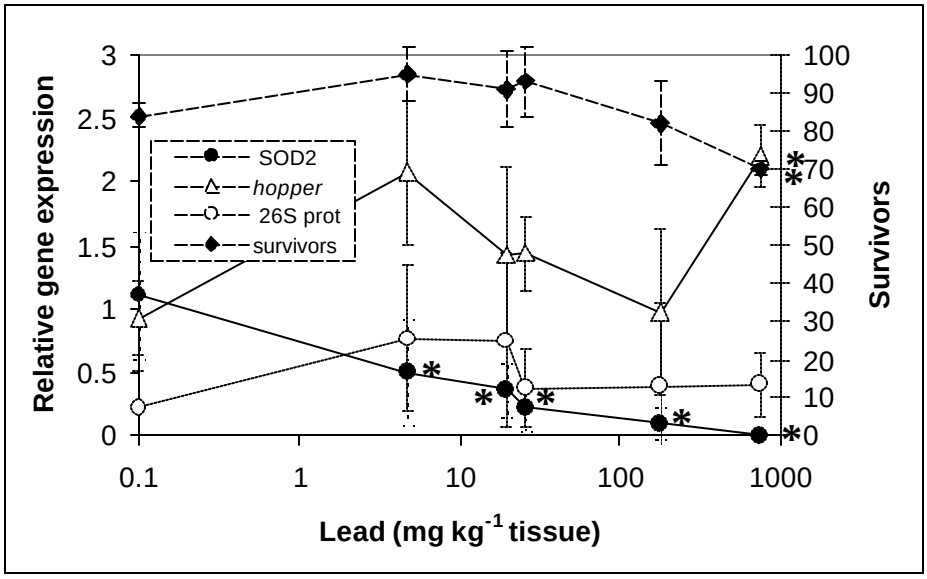
* Significantly different from control (William's test P=0.05).

Figure 5.b.1.D. Increased expression of QM protein and b4 genes are found at levels of lead where a significant decrease in survival occurs.



Each point is the average of three replicate exposures. Expression was normalized to 18S rRNA copies and is relative to unexposed controls. No significant change in actin expression was detected. Data points marked with asterisks are significantly different from controls (Student's ttest, $P<0.05$).

Figure 5.b.1.E. Log linear relationship of lead tissue levels to expression of superoxide dismutase 2 to increasing lead concentrations.



Each point is the average of three replicate exposures. Expression was normalized to 18S rRNA copies and is relative to unexposed controls. No significant change in 26S protease regulatory subunit expression was detected. Data points marked with asterisks are significantly different from controls (Student's ttest, $P<0.05$).

Equation 1

$$\begin{bmatrix} n_1(t+1) \\ n_2(t+1) \\ n_3(t+1) \\ n_4(t+1) \\ .. \\ n_m(t+1) \end{bmatrix} = \begin{bmatrix} F_1 & F_2 & F_3 & F_4 & .. & F_m \\ S_1 & 0 & 0 & 0 & .. & 0 \\ 0 & S_2 & 0 & 0 & .. & 0 \\ 0 & 0 & S_3 & 0 & .. & 0 \\ .. & .. & .. & .. & .. & 0 \\ 0 & 0 & 0 & 0 & S_{m-1} & S_m \end{bmatrix} \begin{bmatrix} n_1(t) \\ n_2(t) \\ n_3(t) \\ n_4(t) \\ .. \\ n_m(t) \end{bmatrix}$$

Equation 2

$$N_t = N_0 l^t$$

Equation 3

$$s_x = \left(\frac{count(x+1)}{count(x)} \right)$$

Equation 4

$$f_x = \left(\frac{offspringcount(x+1)}{count(x)} \right)$$

Equation 5

$$\Delta l = l^e - l^c$$

Equation 6.

$$l^e - l^c \approx \sum_{ij} \left(a_{ij}{}^e - a_{ij}{}^c \right) \frac{\partial l}{\partial a_{ij}} \Bigg|_{\left(A^e + A^c \right)/2}$$

Table 5.b.2.A. Contributions to change in lambda.
Control = food type 1, Experimental = food type 2

	Contribution to change in lambda	
Age class	by survival	by fecundity
0-1 week	0	0.02
1-2 weeks	-0.04	-0.01
2-3 weeks	-0.01	-0.02
3-4 weeks	0	-0.01
>4 weeks	-0.01	0

Table 5.b.1.B. Lambda for each treatment.

	Food	
Sediment	normal (1x)	double (2x)
0%	1.543	1.624
3%	1.472	1.532
6%	1.322	1.397

Table 5.b.1.C. Change in lambda

Control	Treatment	Change in lambda
(0%, 1x)	(3%, 1x)	-0.071
(0%, 2x)	(3%, 2x)	-0.092
(0%, 1x)	(6%, 1x)	-0.221
(0%, 2x)	(6%, 2x)	-0.227
(3%, 1x)	(6%, 1x)	-0.150
(3%, 2x)	(6%, 2x)	-0.135
(0%, 1x)	(0%, 2x)	0.081
(3%, 1x)	(3%, 2x)	0.060
(6%, 1x)	(6%, 2x)	0.075

Figure 5.b.1.A. *Leptocheirus* survival and reproduction curves during exposure to BRH**.**

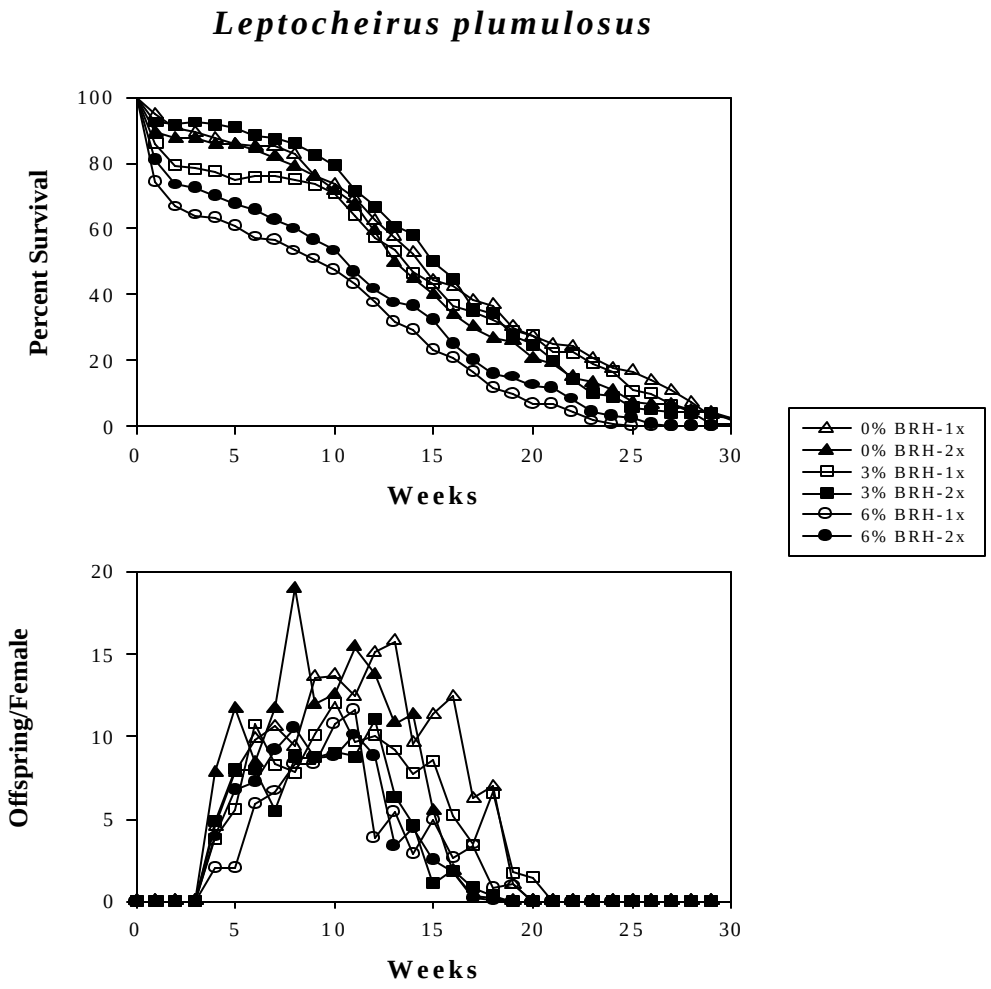


Figure 5.b.1.B. Contributions to the change in lambda by age-specific survival rates for combinations at normal (1x) food ration.

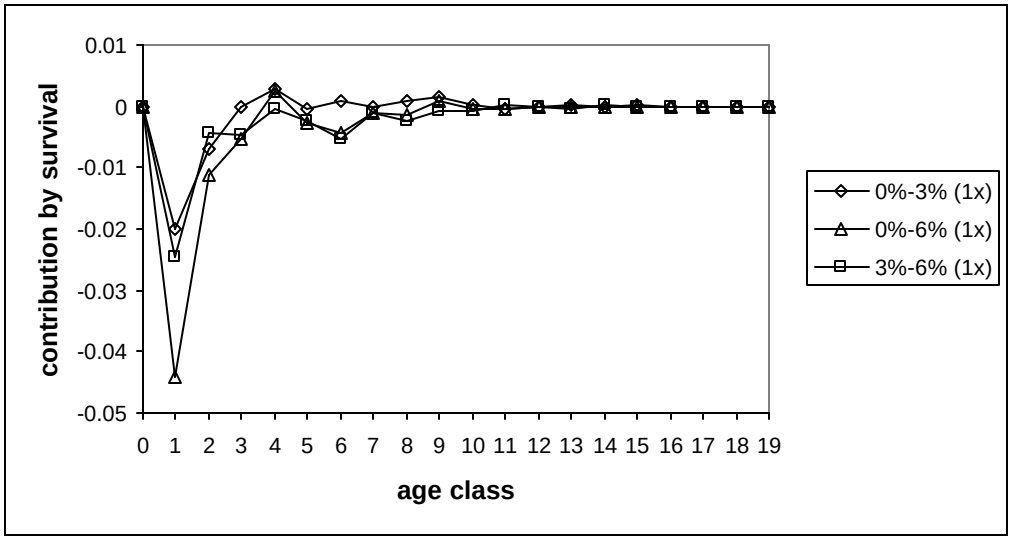


Figure 5.b.1.C. Contributions to the change in lambda by age-specific fecundities for combinations at normal (1x) food ration.

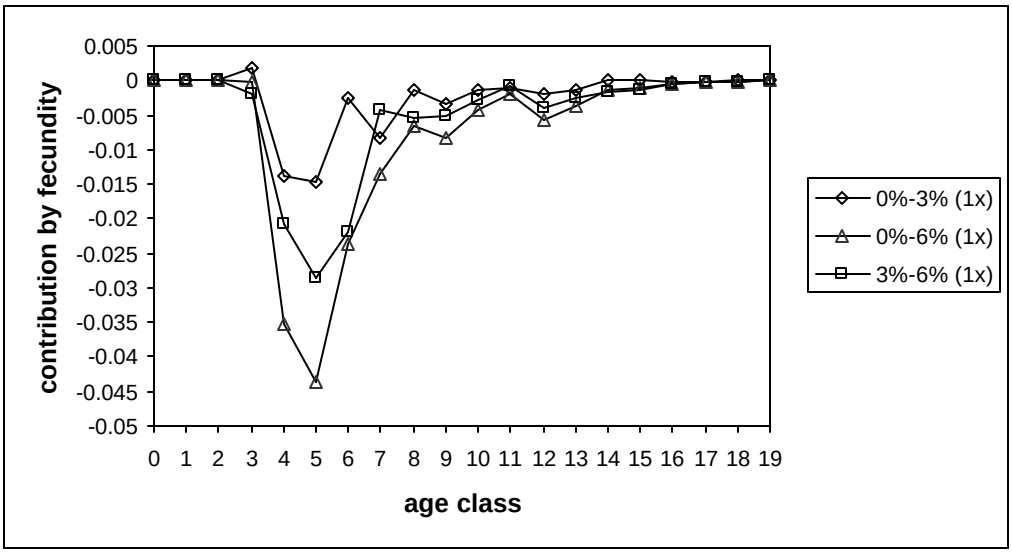


Table 5.b.3.A. Concentrations (µmol/L) of TNT and TNT daughter compounds in aqueous solutions used in single-compound exposures.

Compound	Target	Measured	24h-aged		
			% loss	% 4ADNT	% DANTs
TNT	5.0	2.4 ± 0.3	53.3 ± 6.4	73.1 ± 10.5	26.9 ± 10.5
	7.5	3.5 ± 0.2	62.4 ± 8.3	83.6 ± 4.9	16.4 ± 4.9
	11.0	5.1 ± 0.4	66.5 ± 7.6	79.4 ± 7.8	20.6 ± 7.8
	16.9	7.5 ± 0.5	63.5 ± 3.0	89.2 ± 3.3	10.8 ± 3.3
	25.3	11.3 ± 0.2	67.9 ± 1.3	88.8 ± 0.4	11.2 ± 0.4
			% loss	% DANTs	
2ADNT	10.0	8.1 ± 0.2	19.0 ± 14.2	25.0 ± 8.2	nd
	15.0	12.2 ± 0.3	25.1 ± 13.0	24.7 ± 9.0	nd
	22.5	17.9 ± 0.4	16.8 ± 23.8	16.8 ± 10.3	nd
	33.8	28.7 ± 0.1	17.5 ± 24.7	10.9 ± 5.5	nd
	42.2	36.3 ± 1.5	14.1 ± 14.5	17.0 ± 8.3	nd
			% loss		
2,4DANT	40.0	25.6 ± 1.9	19.5 ± 21.7	nd	nd
	80.0	66.6 ± 3.6	14.4 ± 20.4	nd	nd
	160.0	136.2 ± 3.6	9.8 ± 13.9	nd	nd
	320.0	230.3 ± 10.5	7.8 ± 6.9	nd	nd
	640.0	593.2 ± 4.2	0.9 ± 1.2	nd	nd
			% loss	% ADNB	
TNB	4.0	2.6 ± 0.2	64.6 ± 3.3	87.7 ± 4.2	nd
	8.0	5.7 ± 0.1	70.6 ± 4.1	64.4 ± 4.6	nd
	12.0	8.1 ± 0.5	66.0 ± 5.3	52.7 ± 0.8	nd
	16.0	12.2 ± 1.4	52.8 ± 10.2	37.7 ± 1.6	nd
	24.0	21.3 ± 0.4	48.9 ± 0.4	17.0 ± 4.9	nd

nd = not determined

Table 5.b.3.B. Concentrations (µmol/L) of TNT and TNT daughter compounds in aqueous solutions used in mixture exposures.

Target	Measured	24h-aged						
		% loss	% TNT	% 2ADNT	% 4ADNT	% DANTs	% TNB	% ADNB
7.5	4.4 ± 1.6	44.1 ± 5.7	7.4 ± 10.5	30.4 ± 7.0	1.4 ± 1.9	38.5 ± 6.6	18.1 ± 25.6	4.4 ± 6.2
11.3	7.8 ± 1.1	60.5 ± 0.3	10.1 ± 14.3	26.1 ± 6.8	1.3 ± 1.8	30.7 ± 7.6	28.3 ± 40.0	3.7 ± 5.2
16.9	13.6 ± 1.1	52.5 ± 4.6	10.3 ± 14.6	25.1 ± 7.2	1.7 ± 2.4	30.3 ± 4.8	27.0 ± 34. 5	5.8 ± 8.2
25.3	20.6 ± 1.7	50.7 ± 0.3	10.4 ± 13. 0	27.0 ± 2.1	1.7 ± 2.5	30.2 ± 5.9	27.0 ± 35.1	3.8 ± 5.4
38.0	26.5 ± 1.2	36.5 ± 1.1	14.0 ± 15.7	31.8 ± 2.3	1.6 ± 2.2	36.0 ± 7.1	14.1 ± 12.7	2.7 ± 3.8

Table 5.b.3.C. Ten-day LC50 values for TNT and TNT daughter products in single compounds or mixture exposures.

Compound	LC50 (95% CI) µmol/L	LC50 (95% CI) mg/L
TNT	8.5 (7.7-9.3)	1.9 (1.8-2.1)
2ADNT	16.9 (14.7-19.5)	3.34 (2.9-3.8)
2,4DANT	199.3 (153.4-258.9)	33.3 (25.6-43.2)
TNB	10.2 (9.9-11.7)	2.2 (2.1-2.5)
MIXTURE	14.5 (12.3-17.2)	nd

nd = not determined

Table 5.b.3.D. Concentrations of TNT and TNT daughter compounds in larval midges collected at termination of the single-compound and mixture exposures. Bioconcentration factors (BCF) and the fraction of the each compound comprising the total body burden are presented.

Compound	Water Conc. (µmol/L)	Tissue Conc. µmol/kg	BCF	% 4ADNT	% 2ADNT	% DANTS
TNT	2.4	4.8	2.0	100	0	nd
	3.5	10.1	2.9	100	0	nd
	5.1	13.6	2.7	78	22	nd
	7.5	19.9*	2.6	100	0	nd
2ADNT	8.1	11.9	1.5	nd	45	55
	12.2	17.1*	1.4	nd	58	42
	17.9	11.6*	0.6	nd	36	64
2,4DANT	25.6	27.1	1.1	nd	nd	100
	66.6	41.0*	0.6	nd	nd	100
	136.2	108.9*	0.8	nd	nd	100
	230.3	111.3*	0.5	nd	nd	100
Mixture	4.4	10.3	2.4	49	0	51
	7.8	24.0	3.1	19	39	42
	13.6	42.3*	3.1	0	0	100

nd = not determined

Figure 5.b.3.A. Survival and final biomass at exposure termination of the TNT and TNT daughter products in single compounds and mixture exposures.

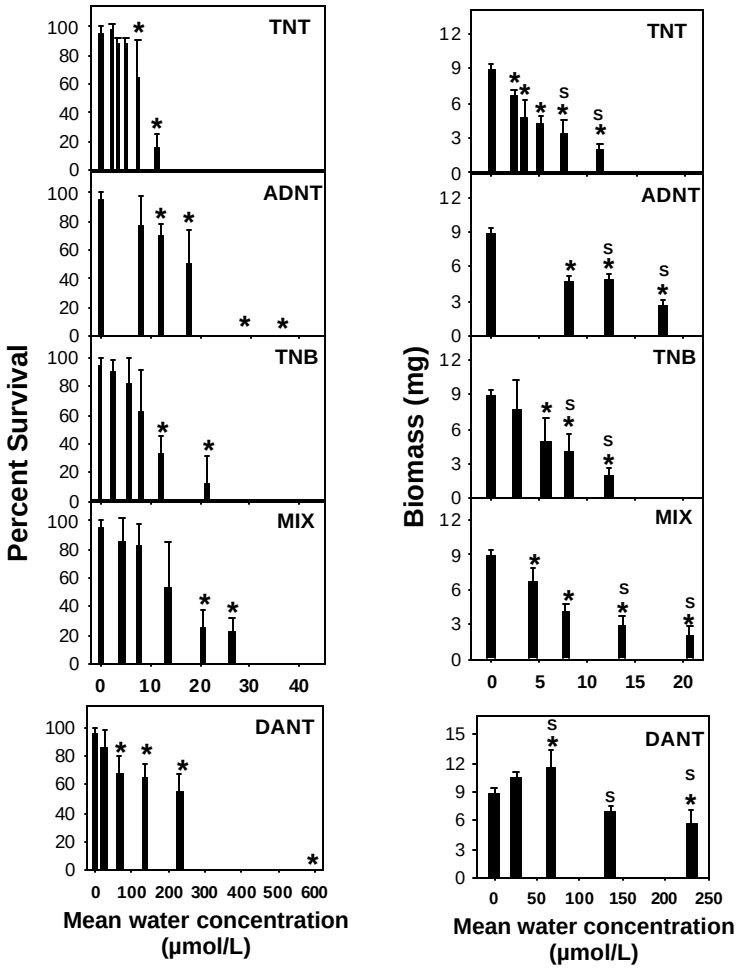


Figure 5.b.3.B. Relationship between survival and sum toxic units calculated for the 10-d mixture exposures.

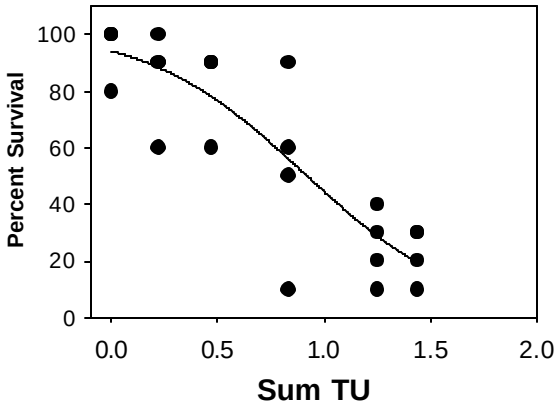


Figure 5.b.3.C. Relationship between mean survival (± 1 standard deviation) and sum molar concentration of nitroaromatic compounds in the tissues of *C. tentans* at termination of the 10-d aqueous single-compound and mixture exposures to TNT and TNT daughter compounds.

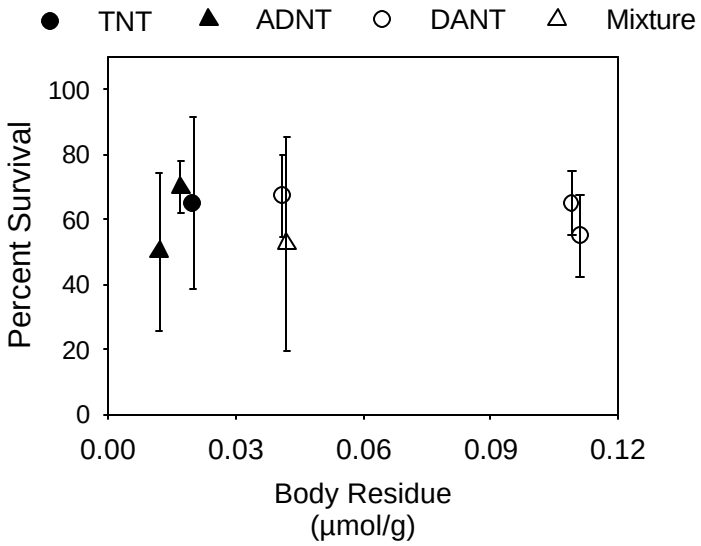


Table 5.b.4.A. Comparative toxicity experiments. Concentration of nitroaromatic compounds in spiked sediments following spiking and mixing.

Expt.	Target (mg/kg)	Target (µmol/kg)	Expected (µmol/kg)	Measured (µmol/kg)							
				TNT	4ADNT	2ADNT	DANTs	TNB	ADNB	Total	% of Expected
TNT	50	220	241	13	85	87	4	nd	nd	188	131
	100	441	396	196	30	48	0	nd	nd	275	69
	200	881	798	460	61	115	0	nd	nd	636	80
	400	1,762	1,763	948	125	268	1	nd	nd	1,342	76
	600	2,643	2,603	650	304	750	8	nd	nd	1,712	66
	800	3,524	3,470	2,668	38	63	0	nd	nd	2,769	80
2ADNT	50	254	261	nd	nd	115	nd	nd	nd	115	44
	100	508	366	nd	nd	301	nd	nd	nd	301	82
	200	1,015	866	nd	nd	658	nd	nd	nd	658	76
	400	2,030	1,960	nd	nd	1,520	nd	nd	nd	1520	78
	600	3,046	2,847	nd	nd	2,124	nd	nd	nd	2,124	94
	800	4,061	3,853	nd	nd	3,266	nd	nd	nd	3,266	85
2,4DANT	150	898	719	nd	nd	nd	199	nd	nd	199	28
	300	1,796	1,453	nd	nd	nd	356	nd	nd	356	25
	600	3,593	3,408	nd	nd	nd	918	nd	nd	918.3	27
	900	5,389	4,427	nd	nd	nd	2,899	nd	nd	2,899	65
TNB	50	235	135	nd	nd	nd	nd	2	53	55	41
	100	469	329	nd	nd	nd	nd	0	146	146	44
	200	939	656	nd	nd	nd	nd	12	341	353	54
	400	1,878	1,398	nd	nd	nd	nd	229	554	784	56
	600	2,817	2,030	nd	nd	nd	nd	624	672	1,296	64
	800	3,756	2,510	nd	nd	nd	nd	560	1,473	2,033	81

(nd = not determined)

Table 5.b.4.B. Mixture experiments. Concentration of nitroaromatic compounds in spiked sediments following spiking and mixing.

Treat. (STU)	Expected (µmol/kg)					Measured (µmol/kg)							
	TNT	2ADNT	2,4DANT	TNB	Total	TNT	2ADNT	4ADNT	DANTs	TNB	ADNB	Total	% Exp
0.25	18	35	84	87	225	0	25	16	54	0	28	123	55
0.5	40	62	218	160	480	0	56	9	113	1	78	257	54
1	95	179	405	349	1,029	12	115	11	233	13	175	559	54
2	137	237	757	606	1,736	49	202	13	431	102	256	1,054	61
3	207	358	855	715	2,134	116	302	109	699	305	217	1,749	82

Table 5.b.4.C. Mixture experiments. Concentration (µmol/kg) of nitroaromatic compounds in spiked sediments following spiking and mixing in single-compound treatments.

Experiment	Target	Expected	Measured							
			TNT	2ADNT	4ADNT	DANTs	TNB	ADNB	Total	% of Exp.
TNT	132	127	0	45	32	15	nd	nd	105	83
	264	238	2	113	71	13	nd	nd	198	83
	529	605	193	134	73	4	nd	nd	404	67
	1,057	1,365	615	182	86	4	nd	nd	886	65
ADNT	254	230	nd	128	nd	42	nd	nd	170	74
	508	542	nd	317	nd	42	nd	nd	359	66
	1,015	1,079	nd	703	nd	32	nd	nd	735	65
	2,030	1,810	nd	1,137	nd	95	nd	nd	1,232	63
DANT	599	487	nd	nd	nd	336	nd	nd	336	69
	1,198	1,177	nd	nd	nd	732	nd	nd	731	62
	2,395	2,475	nd	nd	nd	1,535	nd	nd	1,535	62
	4,790	4,699	nd	nd	nd	2,771	nd	nd	2,779	59
TNB	469	363	nd	nd	nd	nd	1	230	231	64
	939	755	nd	nd	nd	nd	2	426	429	57
	1,878	1,585	nd	nd	nd	nd	57	944	1,001	63
	2,817	2,692	nd	nd	nd	nd	231	1437	1,668	62

nd = not determined

Table 5.b.4.D. Comparative toxicity experiments. Concentration of nitroaromatic compounds in spiked sediments (µmol/g dry wt) at termination of the 10-d exposure.

Experiment	Expected	Measured							
		TNT	4ADNT	2ADNT	DANTs	TNB	ADNB	Total	% Decrease
TNT	144	0	0	31	1	nd	nd	33	83
	396	2	5	7	14	nd	nd	28	90
	798	2	26	19	41	nd	nd	88	86
	1,763	2	278	59	40	nd	nd	380	72
	2,603	2	443	58	79	nd	nd	582	66
	3,470	287	451	166	4	nd	nd	908	67
2ADNT	261	nd		nd	5	nd	nd	5	96
	366	nd	0	nd	0	nd	nd	0	100
	866	nd	17	nd	5	nd	nd	22	97
	1,960	nd	397	nd	66	nd	nd	463	70
	2,270	nd	534	nd	88	nd	nd	622	71
	3,853	nd	654	nd	146	nd	nd	800	75
2,4DANT	719	nd	nd	nd	0	nd	nd	0	100
	1,453	nd	nd	nd	0	nd	nd	0	100
	3,408	nd	nd	nd	36	nd	nd	36	96
	4,427	nd	nd	nd	81	nd	nd	81	97
TNB	135	nd	nd	nd	nd	0	0	0	100
	329	nd	nd	nd	nd	0	0	0	100
	656	nd	nd	nd	nd	0	5	5	99
	1,398	nd	nd	nd	nd	0	53	55	93
	2,030	nd	nd	nd	nd	0	126	126	90
	2,510	nd	nd	nd	nd	nd	nd	nd	nd

(nd = not determined)

Table 5.b.4.E. Ten-day LC50 values for TNT and TNT daughter products in single compounds or mixture exposures.

Experiments	Comparative Toxicity Experiments		Mixture Experiments	
	LC50 (95% CI) µmol/kg	LC50 (95% CI) mg/kg	LC50 (95% CI) µmol/kg	LC50 (95% CI) mg/kg
TNT	248 (205- 298)	56.2 (46.6- 67.7)	260 (212- 318)	58.9 (48.1- 72.1)
ADNT	307 (242- 390)	60.4 (47.6 - 76.7)	175 (147- 209)	34.4 (28.9- 41.0)
DANT	307 (242- 390)	51.2 (40.3- 65.1)	605 (436- 837)	100.9 (72.9- 139.8)
TNB	533 (467- 609)	113.6 (99.5- 129.7)	356 (293- 432)	75.7 (62.3- 92.0)
Mixture	nd	nd	361 (300- 433)	nd

(nd = not determined)

Table 5.b.4.F. Mixture experiments. Individual and sum toxic units (STU) and percent surival (* denotes significant difference from control treatment).

Target STU	Individual TU							Measured STU	% Survival
	Target	Measured							
		TNT	2ADNT	4ADNT	DANTs	TNB	ADNB		
0.25	0.06	0.00	0.14	0.09	0.09	0.00	0.08	0.40	95 ± 6
0.5	0.125	0.00	0.32	0.05	0.19	0.00	0.22	0.78	58 ± 26*
1	0.25	0.05	0.66	0.07	0.38	0.04	0.49	1.68	28 ± 10*
2	0.5	0.19	1.15	0.08	0.71	0.29	0.72	3.14	10 ± 14*
3	0.75	0.45	1.72	0.62	1.16	0.86	0.61	5.42	5 ± 10*

Figure 5.b.4.A. Survival at exposure termination of the TNT and TNT daughter products single compounds and mixture exposures in the comparative toxicity (left) and mixture (right) experiments

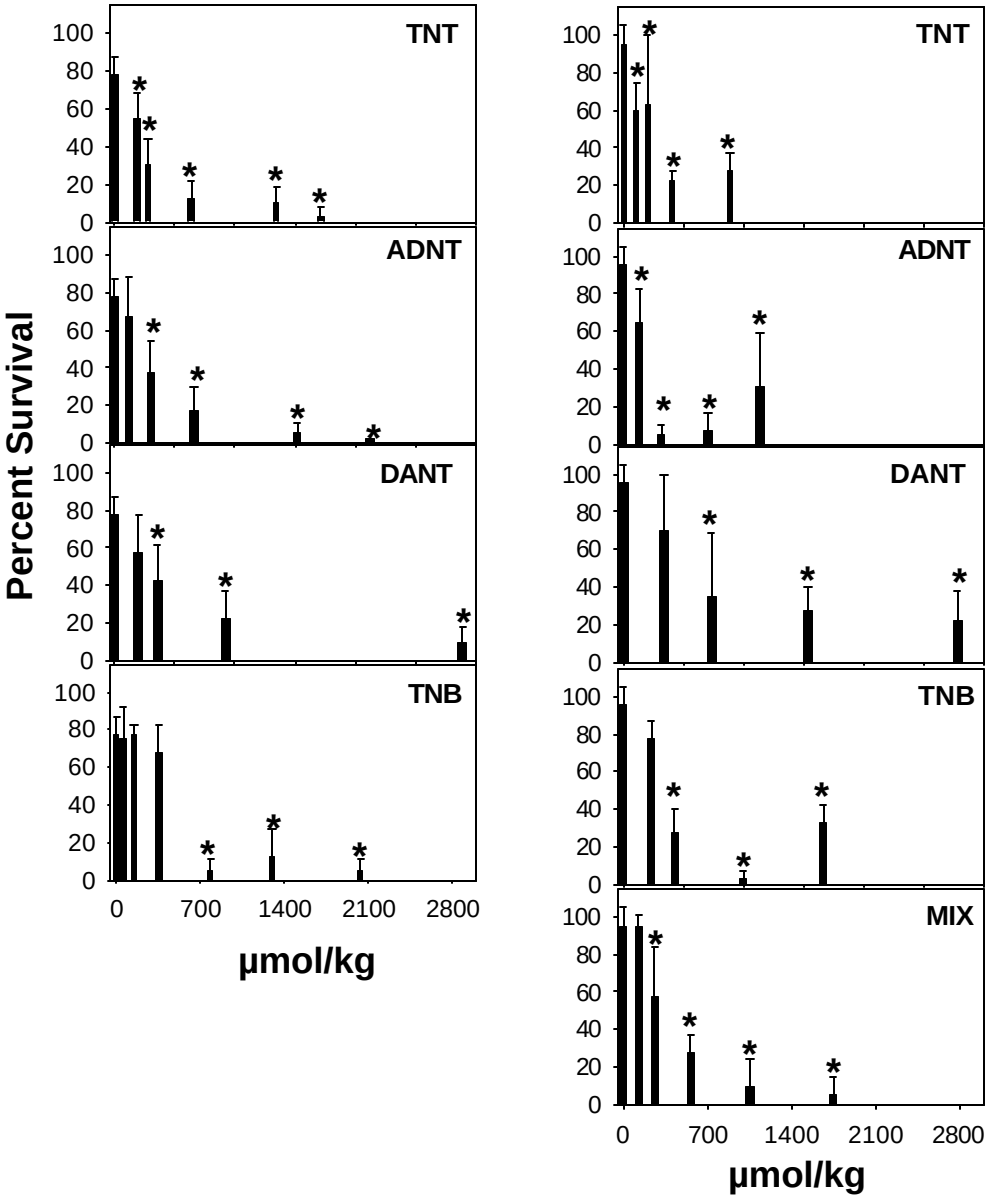


Figure 5.b.4.B. Final individual biomass at termination of the TNT and TNT daughter products single compounds and mixture exposures in the comparative toxicity (left) and mixture (right) experiments.

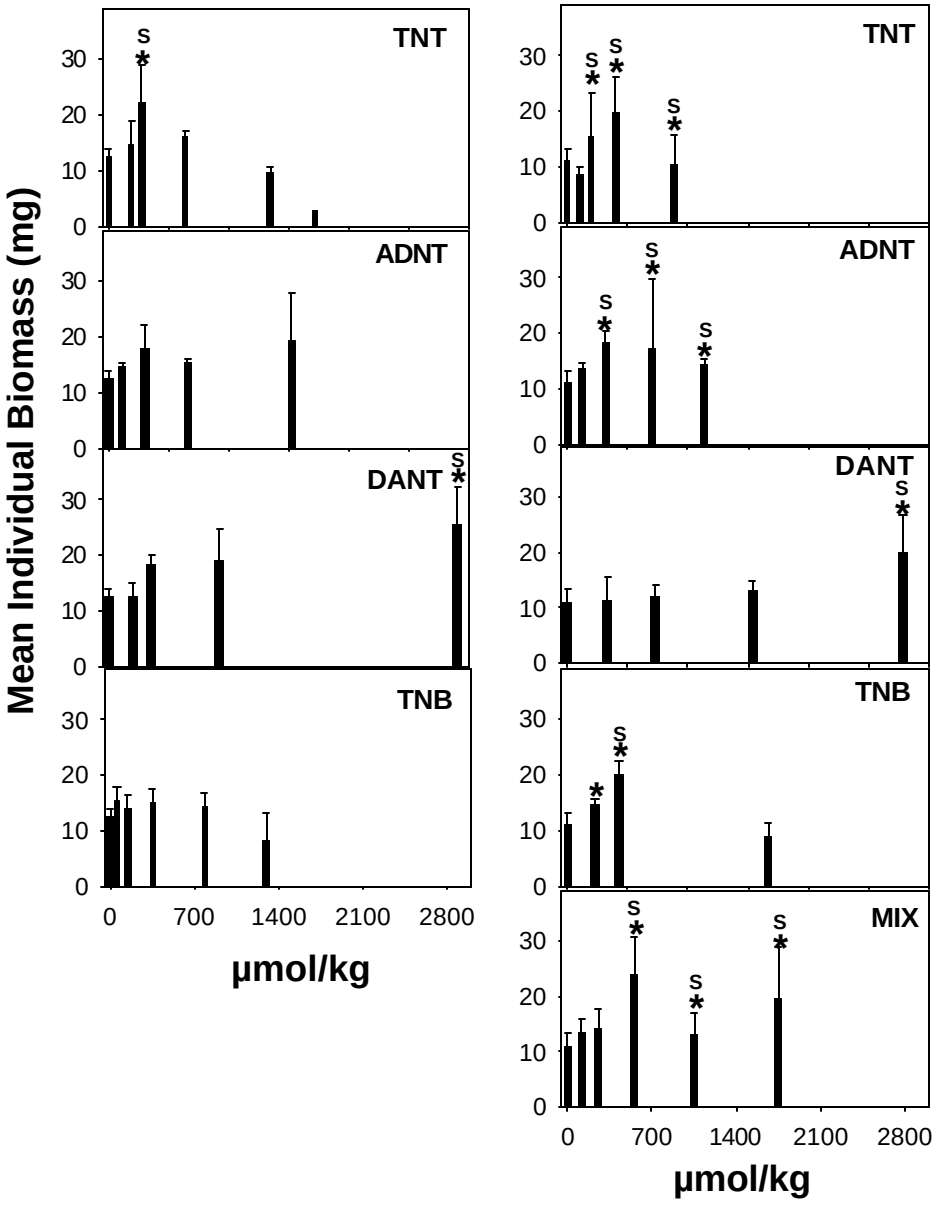


Figure 5.b.4.C. Relationship between survival and sum toxic units calculated for the mixture treatments in the 10-d mixture experiment.

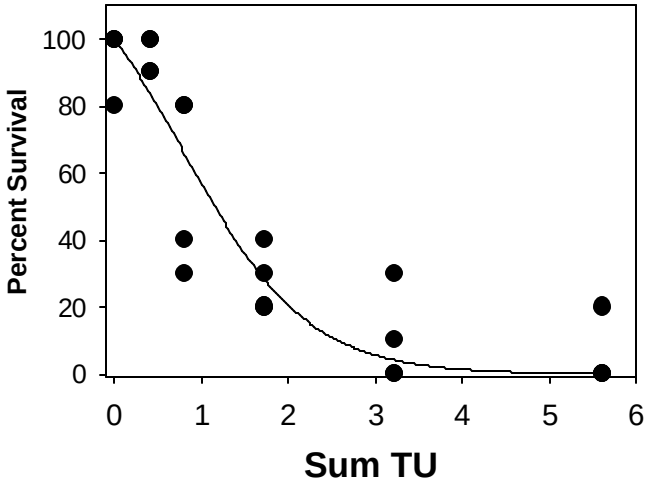


Table 5.b.5.A. Concentrations (µmol/L) of TNB, lead and phenanthrene in single-compound experiments: nominal, measured at day 0 from stock solutions, measured from exposure beakers at day 1 before water exchange and average of day 0 and day 1 concentrations.

Compound	Concentration (µmol/L)				
	Target	measured			
		Stock	Day 1	Mean	Percent decrease
TNB	6	2.66	1.83	2.24	31.1
	9	3.66	2.94	3.30	19.6
	12	5.42	4.20	4.81	22.4
	15	6.94	5.47	6.21	21.2
	25	11.78	10.87	11.33	7.8
Lead	0.1	0.080	0.083	0.081	-3.6
	0.3	0.358	0.211	0.284	41.2
	0.6	0.681	0.676	0.679	0.7
	1.2	1.382	0.855	1.118	38.1
	2.4	2.952	1.536	2.244	48.0
Phenanthrene	0.25	0.20	0.09	0.14	54.8
	0.5	0.28	0.10	0.19	63.6
	0.75	0.57	0.25	0.41	56.3
	1	0.72	0.23	0.47	68.4
	1.5	0.80	0.31	0.55	61.3
	2	1.19	0.74	0.96	37.4
	3	2.38	1.02	1.70	57.0

Table 5.b.5.B. Concentrations (µmol/L) of TNB, lead and phenanthrene in mixture experiments: nominal, measured at day 0 from stock solutions , measured from exposure beakers at day 1 before water exchange and average of day 0 and day 1 concentrations.

Treatment (Sum TU)	Concentrations (µmol/L)		
	TNB	Pb	Phe
	Target		
0.25	0.88	0.018	0.11
0.5	1.75	0.036	0.21
1	3.50	0.072	0.42
2	7.01	0.143	0.85
3	10.51	0.215	1.27
	Measured – stock		
0.25	0.73	0.013	0.11
0.5	1.69	0.028	0.20
1	3.64	0.043	0.39
2	5.76	0.104	0.89
3	14.27	0.157	1.32
	Measured – day 1		
0.25	0.00	0.007	0.02
0.5	0.00	0.014	0.03
1	1.78	0.025	0.07
2	3.26	0.059	0.14
3	4.14	0.089	0.19
	Mean		
0.25	0.37	0.010	0.07
0.5	0.84	0.021	0.12
1	2.71	0.034	0.23
2	4.51	0.081	0.52
3	9.20	0.123	0.76
	Percent decrease		
0.25	100	44.2	79.67
0.5	100	50.9	41.11
1	51.03	40.3	41.34
2	43.44	43.5	41.96
3	71.01	43.1	42.70

Table 5.b.5.C. Nominal 10-d LC50 values from single compound experiments.

Experiment	*Preliminary Experiments*		*Mixture Experiments*	
	LC50 (95% CI) µmol/L	LC50 (95% CI) µg/L	LC50 (95% CI) µmol/L	LC50 (95% CI) µg/L
TNB	10.2 (9.9-11.7)	2239 (2173-2568)	3.02 (2.1-4.5)	643 (434-951)
Lead	0.16 (0.13-0.19)	44 (26-52)	0.13 (0.09-0.18)	26 (18-37)
Phenanthrene	1.27 (1.13-1.42	226 (201-253)	0.44 (0.36-0.54)	78 (64-96)

Table 5.b.5.D. Target and measured Toxic Units (TUs) for each treatments in the mixture experiment.

Treatment (Target STU)	Target Individual TUs	Mesured individual TUs			STUs
		TNB	Pb	Phe	
0.25	0.08	0.12	0.08	0.15	0.35
0.5	0.17	0.28	0.16	0.26	0.71
1	0.33	0.90	0.27	0.52	1.69
2	0.66	1.49	0.65	1.17	3.31
3	1	3.05	0.98	1.72	5.75

Figure 5.b.5.A. Mean percent survival of amphipods exposed to aqueous solutions of TNB, lead (Pb) and phenanthrene (PHE)**.**

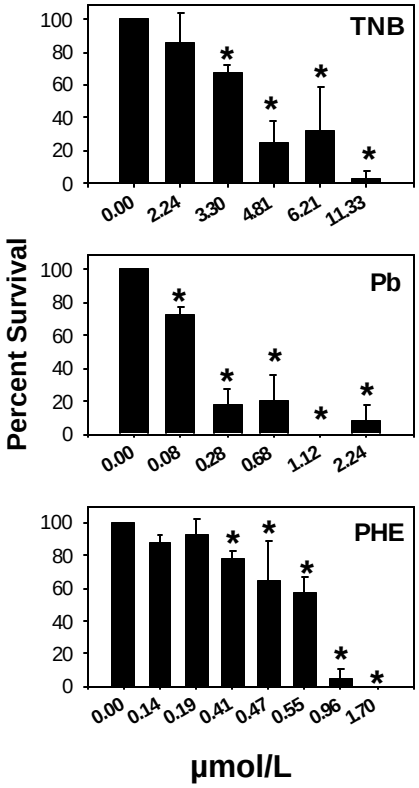


Figure 5.b.5.B. Percent survival a function of the sum toxic units determined for mixture treatments.

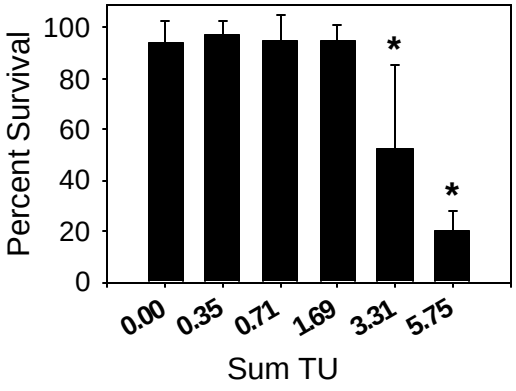


Table 5.b.6.A. Concentration (mg/kg dry wt.) of explosives compounds and some TNT degradation products in the dilution series of 40SD-3 sediment with reference sediment.

Treatment	HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT	TNB
3%	190	1,807	1,366	155	0	0	0
6%	220	2,688	1,582	153	106	0	2
12%	607	6,413	4,126	325	180	4	5
25%	1,077	8,788	7,221	527	218	8	7
50%	3,082	30,588	21,158	1,263	580	27	24
100%	4,733	40,387	31,980	2,477	991	64	38

Table 5.b.6.B. Measured percent dilution (concentration in sample ÷ concentration in 40SD-3 x 100) in the dilution series of SD-3 sediment in reference sediment.

Treatment	HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT	TNB	Mean
3%	4	4	4	6	0	0	0	3
6%	5	7	5	6	11	0	6	6
12%	13	16	13	13	18	6	13	13
25%	23	22	23	21	22	12	18	20
50%	65	76	66	51	59	43	63	60

Table 5.b.6.C. Concentrations and Solubility limits (mg/L) of explosives compounds and some TNT degradation products in the porewater extracted from dilution series of 40SD-3 sediment in reference sediment.

Treatment	HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT	TNB
3%	2.6	79.6	38.5	8.2	7.1	0.16	0.45
6%	2.7	78.7	39.5	9.3	7.4	0.19	0
12%	2.8	78.5	40.2	13.8	10	0.36	0
25%	3	85.4	39.9	20.7	14.09	0.69	0.37
50%	2.9	87.5	39.6	29.9	21.4	2.05	0.28
100%	3.4	87.75	41.9	34.6	29.5	6.87	0.16
Solubility limit	6	130	38	2,800	2,800	>2,800	340

Table 5.b.6.D. Measured percent dilution (concentration in sample ÷ concentration in 40SD-3 x 100) in the porewater extracted from dilution series of 40SD-3 sediment in "reference" sediment.

Treatment	HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT	TNB
3%	76	91	92	24	24	2	281
6%	79	90	94	27	25	3	0
12%	82	89	96	40	34	5	0
25%	88	97	95	60	48	10	231
50%	85	100	95	86	73	30	175

Table 5.b.6.E. Sediment-to-water partitioning coefficient (mg compound/kg sediment ÷ mg compound/L porewater) for explosives compounds and some TNT degradation products dilution series of 40SD-3 sediment in reference sediment.

Treatment	HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT	TNB
3%	0.0137	0.0441	0.0282	0.0528	nd	nd	nd
6%	0.0123	0.0293	0.0250	0.0606	0.0698	nd	nd
12%	0.0046	0.0122	0.0097	0.0425	0.0554	0.0948	nd
25%	0.0028	0.0097	0.0055	0.0393	0.0646	0.0907	0.0531
50%	0.0009	0.0029	0.0019	0.0237	0.0369	0.0750	0.0119

nd = compound not detected in sediment and/or porewater

‘

Table 5.b.6.F. Concentration (mg/L) of explosives compounds and some TNT degradation products in the 40SD-3 sediment porewater dilution series

% of 40SD-3 porewater	Dilution factor	Concentration (mg/L)						
		HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT	TNB
100	0	3.4	87.75	41.9	34.6	29.5	6.87	0.16
8	12.5	0.272	7.020	3.352	2.768	2.360	0.550	0.013
6	16.7	0.204	5.265	2.514	2.076	1.770	0.412	0.010
4	25.0	0.136	3.510	1.676	1.384	1.180	0.275	0.006
3	33.3	0.102	2.633	1.257	1.038	0.885	0.206	0.005
2	50.0	0.068	1.755	0.838	0.692	0.590	0.137	0.003
1	100.0	0.034	0.878	0.419	0.346	0.295	0.069	0.002

Table 5.b.6.G. Toxicity of TNT, TNB, 2,4DANT, RDX and HMX to *Hyalella azteca* in exposures to spiked sediment or water.

Compound	**10-d sediment LC_{50} (mg/kg)**	**10-d water LC_{50}**
TNT	29	2.5 mg/L
TNB	101	nd
2,4DANT	32	nd
RDX	>250	no mortality at 50 mg/L
HMX	>250	no mortality at 5 mg/L

nd = not determined

Table 5.b.6.H. Concentration (mg/kg dry wt.) of explosives compounds and some TNT degradation products in sediments collected from Picatinny Lake in 2002.

Sample	HMX	TNT	RDX	4ADNT	2ADNT	2,4DANT
September Sampling						
Low	0	1.3	0	0	0	0
Medium	0	0	0	0	0	0
High	0	0	0	0	0	0
November Sampling						
SD-3	0	0.4	0	0.2	0	0

Figure 5.b.6.A. Mean percent survival of amphipods and oligochaetes exposed to a dilution series of 40SD-3 sediment.

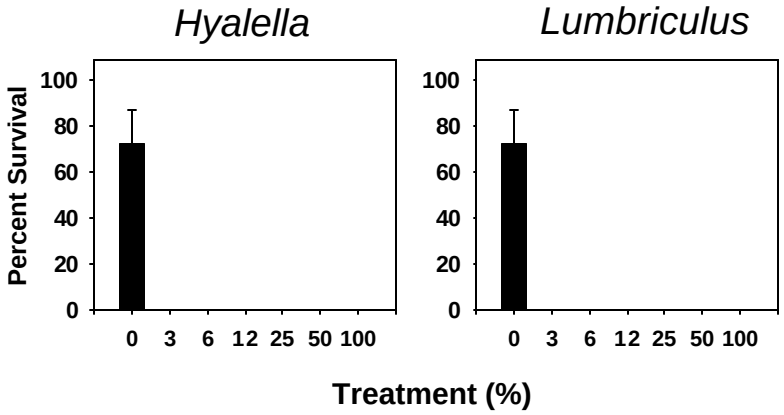


Figure 5.b.6.B. Mean percent survival of amphipods exposed to a dilution series of 40SD-3 sediment porewater.

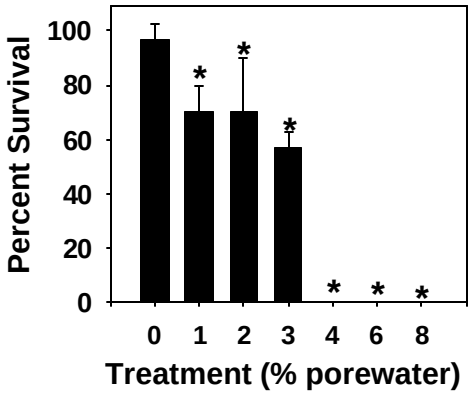


Table 5.b.7.A. Water concentrations during the *C. tentans* 4-d exposure expressed as TNT-molar equivalents (LSC) or sum molar concentration of TNT and 4ADNT (HPLC).

Target (mg/L)	Target (µmol/L)	Measured (µmol/L) LSC Mean	LSC 24-h % decrease	HPLC Mean	HPLC 24-h % decrease	HPLC % 4ADNT	HPLC % LSC
0	0	0		0			
0.25	1.1	0.92 ± 0.09	9.7 ± 12.5	0.60 ± 0.40	63.7	93.1	65.3
0.5	2.2	2.06 ± 0.06	7.7 ± 3.6	1.01± 0.50	51.8	76.7	48.9
1	4.4	3.81 ± 0.24	10.9 ± 4.8	2.74 ± 1.59	58.0	62.0	72.0
2	8.8	7.43 ± 0.72	11.3 ± 12.1	6.27 ± 3.53	56.9	87.3	84.4
4	17.6	13.69 ± 1.36	12.7 ± 12.2	10.48 ± 4.16	43.8	66.5	76.5
6	26.4	20.43 ± 10.31	5.6 ± 9.6	15.61 ± 7.46	50.5	51.1	76.4

Table 5.b.7.B. Tissue concentrations at termination of the *C. tentans* 4-d exposure expressed as TNT-molar equivalents (LSC) or sum molar concentration of TNT and its breakdown products (HPLC) and corresponding bioconcentration factors (BCF).

Treatments	Survival	LSC Body Residue (µmol/g)	LSC BCF	HPLC Body Residue (µmol/g)	HPLC BCF	HPLC % LSC
0	94.0 ± 8.9					
1.1	87.5 ± 12.6	0.11 ± 0.02	123 ± 17	0.012	20.5	10.9
2.2	82.5 ± 17.1	0.27 ± 0.08	129 ± 36	0.010	10.3	3.7
4.4	87.5 ± 9.6	0.45 ± 0.09	117± 24	0.015	5.3	3.3
8.8	85.0 ± 9.6	0.75 ± 0.19	101± 26	0.053	8.5	7.1
17.6	27.5 ± 28.7	1.25 ± 0.04	85 ± 12	0.178	16.9	14.2
26.4	0	Nd	Nd	Nd	Nd	nd

Nd = not determined

Table 5.b.7.C. Four-day LC50 and LR50 values expressed as TNT-molar equivalents (LSC) or sum molar concentration of TNT and its breakdown products (HPLC).

	LC50 (µmol/L)	LR50 (µmol/g)
LSC	10.55 (9.11- 12.22)	1.05 (0.97 - 1.14)
HPLC	8.54 (7.2- 10.13)	0.12 (0.10 - 0.14)

Figure 5.b.7.A. Percent of the total body residue comprised of TNT and TNT breakdown products in the tissue of C. tentans at termination of a 4-d aqueous exposure to TNT.

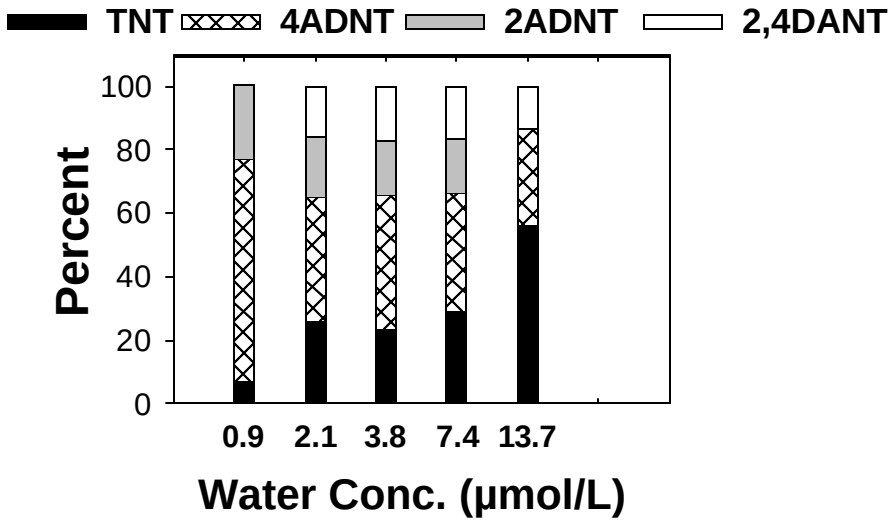


Figure 5.b.7.B. Percent survival of *C. tentans* at termination of a 4-d aqueous exposure to TNT as a function of body residue determined as TNT-molar equivalents (LSC) or sum molar concentration of TNT and its breakdown products (HPLC).

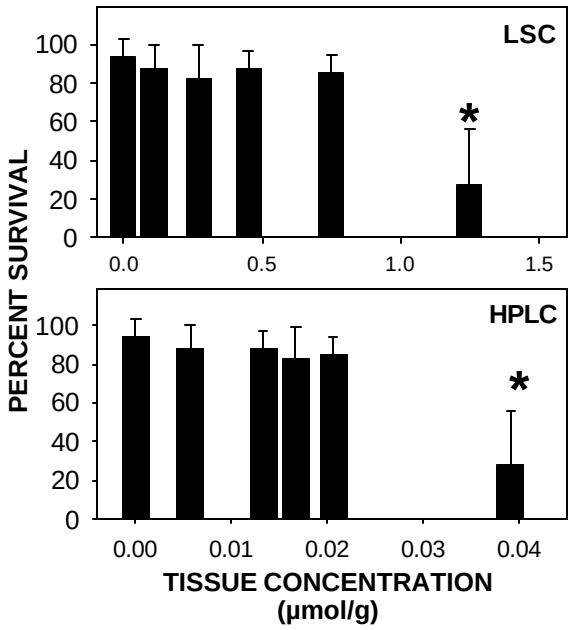


Table 5.b.8.A. Water concentrations during the *L. plumulosus* 8-d exposure expressed as TNT-molar equivalents (LSC) or sum molar concentration of TNT and 4ADNT (HPLC).

Target		Measured					
mg/L	µmol/L	LSC		HPLC			
		Mean	24-h % decrease	Mean	% 4ADNT	24-h % decrease	HPLC/LSC
0.25	1.1	1.08 ± 0.03	5.9 ± 6.4	0.52 ± 0.40	37.4 ± 34.2	61.7 ± 20.5	47.6 ± 34.6
0.5	2.2	1.98 ± 0.04	8.7 ± 4.6	0.99 ± 0.70	28.7 ± 34.1	65.3 ± 10.5	50.2 ± 34.7
1	4.4	4.32 ± 0.29	7.4 ± 3.8	2.48 ± 1.35	17.3 ± 21.8	57.8 ± 9.7	61.6 ± 30.3
2	8.8	7.90 ± 0.15	0.3 ± 2.1	5.29 ± 2.02	8.1 ± 7.5	38.1 ± 10.9	65.7 ± 24.0
4	17.6	15.43 ± 0.06	0	12.58 ± 2.28	1.1 ± 1.6	22.7 ± 5.0	90.4 ± 16.7
6	26.4	23.28 ± 0.10	1.1 ± 0.5	21.61 ± 0.71	0.4 ± 0.6	4.5 ± 6.9	103.4 ± 2.5

Table 5.b.8.B. Toxicokinetic parameters derived from the *L. plumulosus* 8-d exposure: uptake rate (K_u), elimination rate (K_e), elimination half-life ($t_{1/2}$), time for 95% steady state (TSS_{95}) and bioconcentration factor (BCF). * = *p* value for the estimate > 0.1.

Target (µmol/L)	K_u (ml g^{-1} h^{-1})	K_e h^{-1}	$t_{1/2}$ (h)	TSS_{95} (h)	BCF (K_u / K_e)	Measured BCF
1.1	5.3 ± 1.9	0.027 ± 0.011	26	110	196	192
2.2	2.9 ± 0.6	0.010 ± 0.036	69	299	290	252
4.4	5.1 ± 2.6	0.024 ± 0.015	29	124	212	183
8.8	1.3 ± 0.7	0.005 ± 0.007*	139	ND	260	203

Table 5.b.8.C. Four-day LC50 and LR50 values expressed as TNT-molar equivalents (LSC) or sum molar concentration of TNT and its breakdown products (HPLC).

Day	LC50		LR50
	mg/L	µmol/L	µmol/g
Day 4	2.5 (2.5-2.5)	10.9 (10.8 - 11.0)	1.12 (1.11 - 1.13)
Day 8	1.4 (1.3-1.4)	6.1 (5.9 - 6.3)	1.07 (0.96 - 1.20)

Figure 5.b.8.A. Accumulation of TNT molar-equivalents in the tissues of *L. plumulosus* over time during 8-d exposures to different TNT aqueous treatments. Curves represent model predicted values.

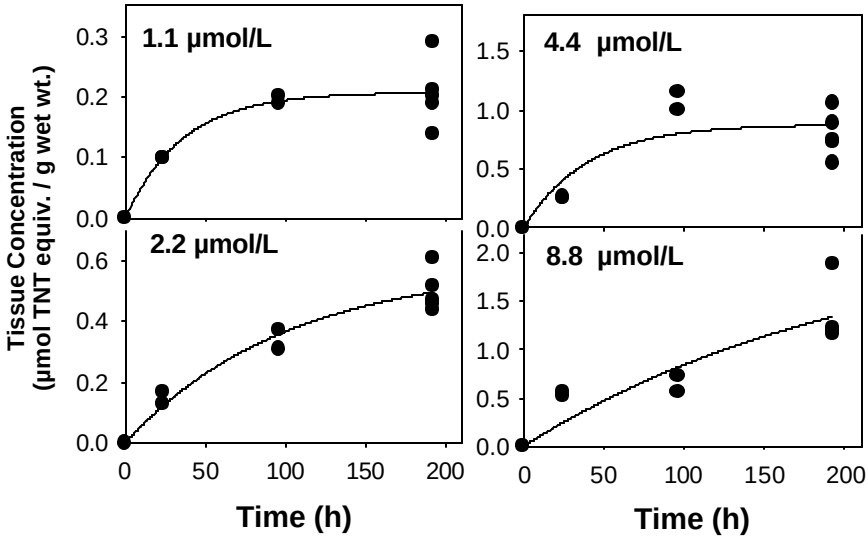


Figure 5.b.8.B. Percent survival of *L. plumulosus* exposed to different TNT aqueous treatments at days 2, 4 and 8. * indicates significant difference from the control.

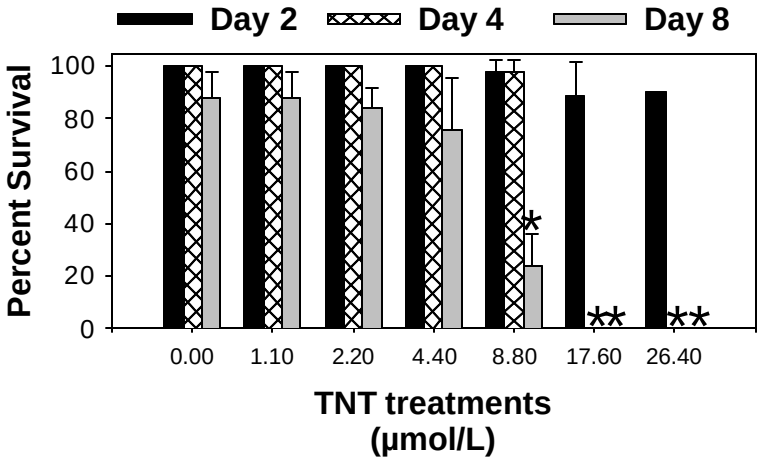
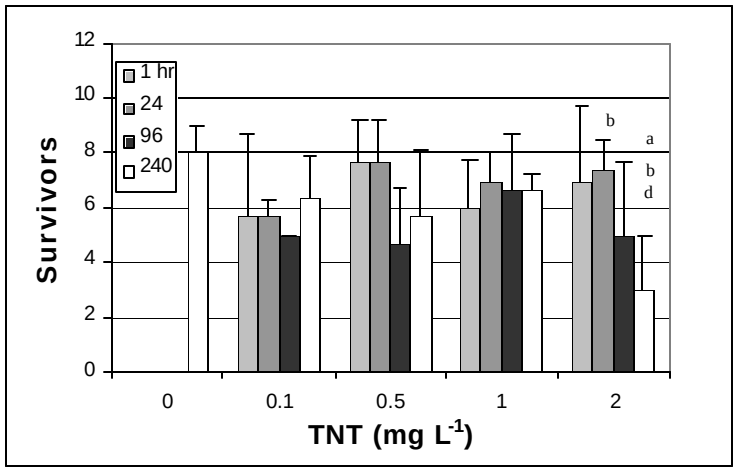


Figure 5.b.9.1. Decreases in *Chironomus tentans* growth and survival are dependent upon TNT exposure concentration and duration.
A. The effect of aqueous TNT exposures on survival at 1, 24, 96, and 240 hrs. B. The effect of aqueous TNT exposures on growth as average wet weight of animals at 1, 24, 96, and 240 hrs. Means significantly different from 240-hr control are denoted with an "a". Means significantly different from 0.1 mg L^{-1} TNT exposure at the same time point are denoted with a "b". Means significantly different from 0.5 mg L^{-1} TNT exposure at the same time point are denoted with a "c". Means significantly different from 1.0 mg L^{-1} TNT exposure at the same time point are denoted with a "d". Means significantly different from 2.0 mg L^{-1} TNT exposure at the same time point are denoted with an "e". Means significantly different from same exposure at the 1-hr time point are denoted with an "f'. Significant differences by Student's t-test, $p<0.05$.

A



B

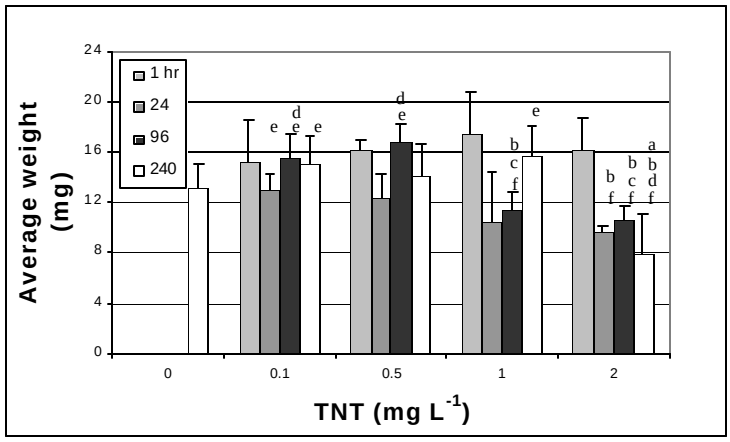
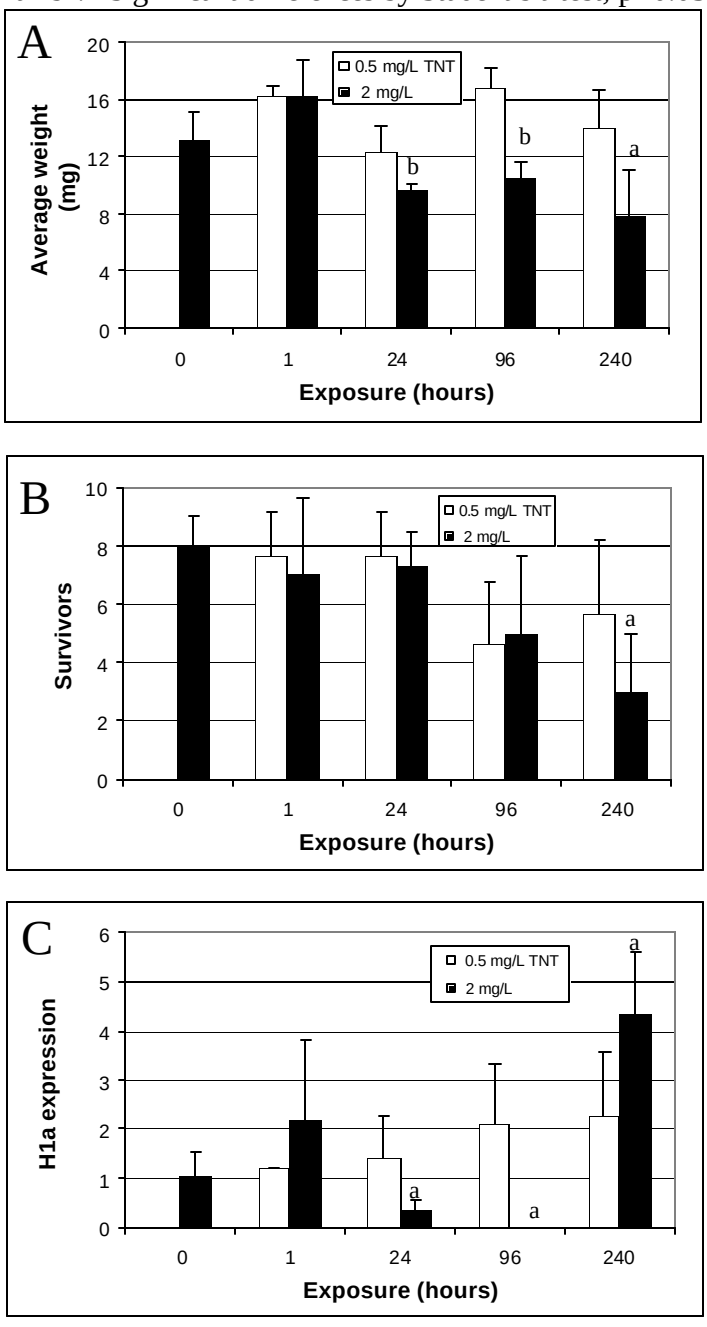


Fig 5.b.9.2. Effect of TNT on histone H1a gene expression, survival, and growth over time.

A. Effect of TNT on weight over time. B. Effect of TNT on survival over time. C. Effect of TNT on histone H1a gene expression over time. Means significantly different from 240-hr control are denoted with an "a". Means significantly different from 0.1 mg L^{-1} TNT exposure at the same time point are denoted with a "b". Significant differences by Student's t-test, $p<0.05$.



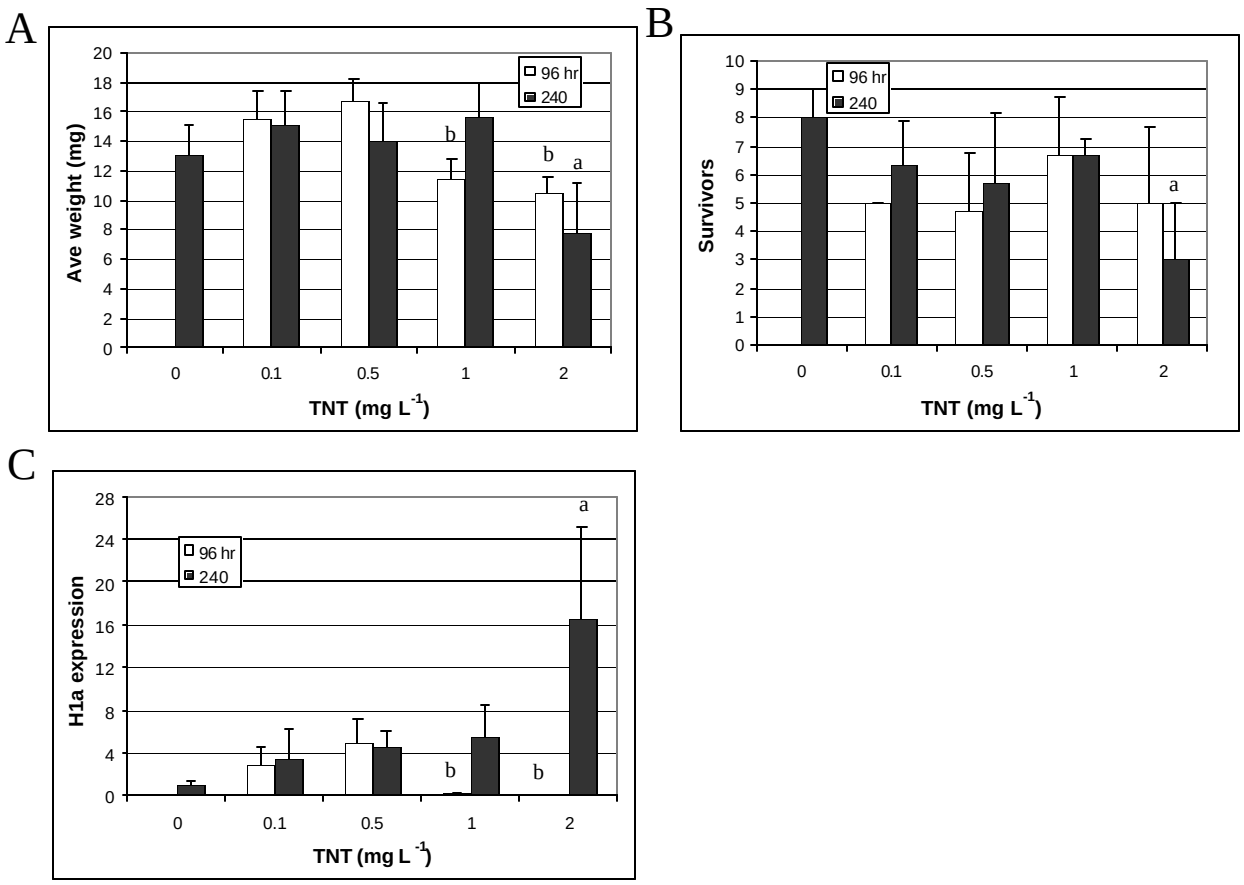


Figure 5.b.9.3. Histone 1a expression levels correlate with both growth and survival at different levels of TNT.

Means significantly different from 240-hr control are denoted with an "a". Means significantly different from 96-hr 0.1 mg L^{-1} TNT exposure are denoted with a "b". Significant differences by Student's t-test, $p<0.05$.

Table 5.b.9.1. Histone h1a expression is affected by exposure to PAHs, a pesticide, and metals in *C. tentans*.

Expression was determined in 16-hr exposures and normalized to 18S rRNA.

Chemical	Exposure (µmole L^{-1})	H1a expression relative to control	H1a normalized to µmole L^{-1} exposure toxicant	H1a expressionn ormalized to toxic dose (LC_{50})	LC_{50} (µmole L^{-1})	water quality criteria[g] (µmole L^{-1})
DDT	0.00056	14.0±5.0 [a]	24850	84	0.0034 [b] (10-d exposure)	0.0031
Phenanthrene	0.14	25.2 ±7.3 [a]	179.6	227.8	1.27 [c] (10-d)	na
Fluoranthene	0.15	2.3± 0.9	ns	13.3	1.12 [c] (10-d)	na
Cd as $CdCl_2$	0.55	4.3±1.9[a]	7.9	27.3	3.46 [d] (14-d)	0.018
Cu as CuCl	0.55	2.4±0.5 [a]	4.4	23.5	5.34 [e] (12-d)	0.205
Zn as $ZnCl_2$	0.917	1.53±0.41	ns	9	5.50 [f] (20-d)	1.84
Acetone		1.0±0.2				

ns=not significantly different from control

na=not available

a. Significantly different from acetone control, Student's t-test $p<0.05$.
b. Phipps, *et al*., 1995, Hoke, *et al*., 1997.
c. Lotufo, unpublished
d. Suedel, *et al*. 1997.
e. Nebeker, *et al*., 1984.
f. Sibley, *et al*., 19967USEPA, 2002